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IMPROVED GAS ANALYSIS IN METABOLIC EXPERIMENTS

SECOND COMMUNICATION

BY

C. ROMIJN AND W. LOKHORST

1. INTRODUCTION

In a first communication, ROMIJN and LOKHORST (1959) gave a brief review of different methods of gas analysis, generally used in experiments on respiratory metabolism of man and animals.

The method based on thermal conductivity of respiratory gases was described with its possibilities, its limitations and adaptations to different conditions.

In recent years, however, many details of the technique have been improved considerably, whereas the method of calculating the results and subsequent calibration of the apparatus have been modified in order to facilitate the interpretation of the diaferometer recordings.

Particularly the new method of calibration increases the accuracy of the data on respiratory metabolism on account of its being independent of the respiratory quotient.

2. THE DIAFEROMETER

For general description and theoretical background of the "diaferometer" we refer to the above mentioned paper of ROMIJN and LOKHORST in which the most important literature has been critically reviewed. Some modifications in the gas supply have been introduced and may be understood from Fig. 1.

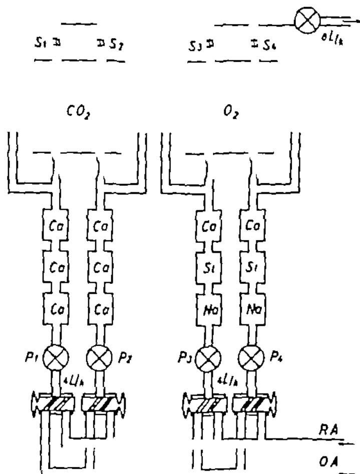


Fig 1

Gas supply to the diaferometer

The main difference with the earlier method lies in the way of purging the channels of the measuring block. The reference gas (outdoor air) as well as the respiration gas is pumped into 4 open tubes after removal of its water vapour and (or) its carbon dioxide. The membrane pumps (P_1 P_2 P_3 P_4) have a capacity of 4 litres per hour. The gas, flowing through the channels of the diaferometer itself is withdrawn from the open tubes with a speed of 2 litres per hour. This modification proved to be very satisfactory on account of the fact that the flow rate in the channels remains much more constant being completely independent of mesh size in the absorption vessels. Moreover the 4 needle valves (S_1, etc)

allow the adjustment of the individual gas streams in order to compensate inevitable small differences in resistance of the platinum wires. Even considerable fluctuations of the total gas flow through all diaferometer channels do not result in a change of electrical output from the bridges. In the CO_2-diaferometer dry respiration gas is compared with dry outdoor air in the O_2-diaferometer dry CO_2-free respiration air with dry CO_2-free outdoor air

Modifications of greater importance have been made in the wiring of the apparatus. First of all the wire current has been elevated from 50 mA to 60 mA and the chemical sensitivity of the instrument has therefore been increased considerably (by about 56 per Cent.) According to VISSER (195?) the temperature of the platinum wire will be

$$t = 20 + 33 \times I^2 \times 100 = 6^- 5^\circ \text{ C.}$$

This temperature is low enough to guarantee a heat elimination mainly by conduction by the surrounding gas.

It should be borne in mind that the milliamperemeter has been shunted in order to indicate directly the wire current instead of the total bridge current. The battery is a 18 Volt accumulator which is loaded continuously with a current of 1·0 mA.

A very important improvement is the method of measuring and recording the bridge potential. A D C. amplifier of high sensitivity (Pye Cambridge) of the zero indicator type with ranges of 10 100 and 1000 microvolts F.S.D has replaced the *moving* coil galvanometer The input impedance is about *1000 Ohms* and therefore hardly any current *is* withdrawn from the bridge circuit resulting in a much higher sensitivity of the apparatus. The output of the amplifier being 10 *Volts* for F.S.D in all ranges has been put in series with a total resistance of about 8 k Ohms, *of* which 4 k *Ohms* are variable in order to open the possibility of modifying the potential drop per unit of resistance (function of the "span"). By means of a low resistance switch and a suitable series of resistances the potential drop over a part of the total output can be fed into a 1 points millivolt recorder of the continuous balance type. In combination with the microvolt amplifier there is a wide possibility of decreasing or increasing the output of the bridge in decade or smaller steps.

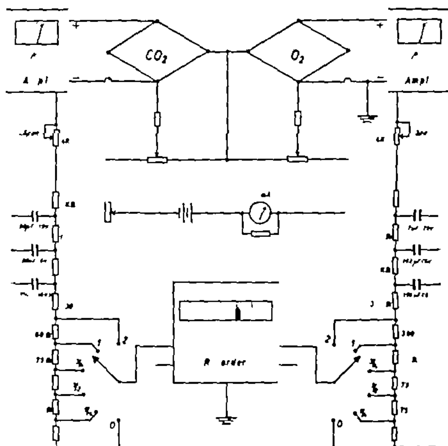


Fig 2.

Wiring diagram of diaferometer and recording system.

The printing outline of the recorder is adapted to the subject under investigation In most cases the input numbers 1 5 and 9 correspond with the signal from the CO_2-diaferometer the numbers 2 6 and 10 with the O_2-diaferometer the numbers 3 7 and 11 with the body temperature of the animal under investigation whereas the numbers 4 8 and 12 are spare inputs which may be used for recording the environmental temperature or humidity

3 CALCULATION OF RESULTS AND CALIBRATION

Definitions ΔCO_2 = Difference in CO_2 percentage of dry respiration air and dry outdoor air

ΔO_2 = Difference in O_2 percentage of dry outdoor air and dry respiration air

The bridge signal (D.C. output) in the CO_2 diaferometer is the sum of the electric unbalances caused by ΔCO_2 and ΔO_2. However the thermal conductivity of CO_2 at the wire temperature is 10.[illegible] times that of O_2 with respect to air (ROMIJN and LOKHORST 1959) and the total output can therefore be expressed by

$$E.M.F._{(CO_2)} = \Delta CO_2 + \frac{1}{10.2} \Delta O_2$$

or approximately

$$E.M.F._{(CO_2)} = \Delta CO_2 + 0.1\ \Delta O_2 \qquad (1)$$

In the O_2-diaferometer the carbon dioxide has been completely absorbed from the outdoor air as well as from the respiration air and the bridge D.C. signal is therefore exclusively the result of a difference in O_2-percentage. This difference is lower than ΔO_2 on account of the CO_2-absorption and a subsequent rise in percentage of the other gases. For outdoor air this rise may be neglected but in the respiration air the O_2-percentage increases by % $O_2/100 \times \Delta CO_2$ or approximately by $0.20 \times \Delta CO_2$.

The bridge output may be expressed by

$$E.M.F._{(O_2)} = \Delta O_2 - 0.20\ \Delta CO_2. \qquad (2)$$

The correct figures for ΔCO_2 and ΔO_2 can be calculated from *(1)* and *(2)* if the diaferometer has been calibrated beforehand with a gas mixture of known composition.

$$\Delta CO_2 = E.M.F._{(CO_2)} - 0.1\ E.M.F._{(O_2)} + 0.[illegible]\ \Delta CO_2$$

or approximately

$$\Delta CO_2 = E.M.F._{(CO_2)} - 0.12\ E.M.F._{(O_2)} \qquad (3)$$

$$\Delta O_2 = E.M.F._{(O_2)} + 0.2\ \Delta CO_2 \qquad (4)$$

In our first communication we have already emphasized the use of respiration gas for calibration under physiological conditions and to avoid in any case the introduction of bomb gases into the channels of the diaferometer.

Suppose we have a calibration mixture of

$$2\ 00 \text{ pCt } CO_2 \quad 18\ 00 \text{ pCt } O_2 \text{ and } 80\ 00 \text{ pCt } N_2$$
$$\Delta CO_2 = 2\ 00 - 0\ 04 = 1\ 96$$
$$\Delta O_2 = 20\ 94 - 18\ 00 = 2.94$$

According to formula (1)

$$E.M.F_{(CO_2)} = 1\ 96 + 0\ 294 = 2\ 254$$

and to formula (2)

$$E.M.F_{(O_2)} = 2\ 94 - 0\ 2 \times 1\ 96 = 2\ 548$$

Thanks to the possibility to adjust the Span of both dia ferometers the millivolt record for the E.M.F (CO_2) can be made to correspond with 225 4 mV and for the E.M.F (O_2) with 254 8 mV Under the above mentioned conditions each 0 01 per Cent CO_2-surplus or O_2-deficit corresponds with 1 mV deflection on the recorder

Needless to say that calibration with such a gas mixture should be performed with the position of the low resistance switch in step ½

Recalculation of ΔCO_2 and ΔO_2 with the formulas (3) and (4) gives

$$\Delta CO_2 = 225\ 4 - 0\ 12 \times 254\ 8 = 1\ 95$$
$$\Delta O_2 = 254\ 8 + 0\ 2 \times 1\ 95 = 2\ 94$$

Calibration of the diaferometer according to the method described makes the instrument a gas analyser insofar as the recorded E.M.F can be interpreted directly into percentages CO_2-surplus and (or) percentages O_2-deficit of the gas in the channels of the measuring bloc. The correct composition of the gas mixture before *entering the apparatus* can be calculated by application of the abovementioned corrections.

The CO_2-production as well as the O_2-*consumption of the subject* under investigation can be calculated from the ΔCO_2 ΔO_2 and the total ventilation per unit of time (V litres/min)

In the case of a respiratory quotient of 1 00 the O_2-consumption

per minute amounts to $V \times \Delta O_2$ and the carbon dioxide production to $V \times \Delta CO_2$, but an R.Q. of 1.00 is an exception. In general the R.Q. is lower than 1.00 and the ventilation air leaving the experimental animal is therefore smaller in volume than the fresh air supplied to the subject. This results in a rise of the nitrogen content of the respiration air above the outdoor figure (79.02 per Cent.) In the above mentioned example of calibration, the situation is as follows

outdoor air	0.04 pCt. CO_2	20.94 pCt. O_2	79.02 pCt. N_2
respiration air	00 pCt. CO_2	18.00 pCt. O_2	80.00 pCt. N_2

If therefore V litres/min leave the respiration chamber or mask or the lungs themselves, an amount of $\frac{80.00}{79.02} \times V$ litres/min of fresh air were supplied to the subject in the same unit of time.

The O_2-consumption may be calculated therefore as

$$\left(\frac{80.00}{79.02} \times V \times \frac{20.94}{100} - V \times \frac{18.00}{100}\right) \text{l/min}$$

or

$$V\left(\frac{80.00 \times 20.94}{79.02 \times 100} - \frac{18.00}{100}\right) \text{l/min},$$

and to the correct figure for ΔO_2 a factor should be added to open the possibility of multiplying directly by V. This correction factor depends on the magnitude of the R.Q. and is zero only in the rare case of an R.Q. = 1.00

In general, the percentage of O_2 by which the ventilation rate (*V l/min*) should be multiplied to find the total O_2-consumption is greater than ΔO_2 and we should like to introduce the name of "metabolic oxygen deficit" for this fictive O_2-deficit.

In our example the "metabolic O_2-deficit" amounts to

$$\frac{80.00}{79.02} \times 20.94 - 18.00 = 3.20 \text{ per Cent},$$

and is considerably higher than the actual O_2-deficit ($\Delta O_2 = 2.94$)

It should be emphasized that for correct results in metabolic

experiments a perfect knowledge of the R Q is absolutely necessary in the case of a calibration procedure as described above and in all other methods of analysis in which the total procentual composition of the respiration gas is determined Moreover it should be borne in mind that the ventilation rate as indicated by a gas meter or flow rator in the stream of respiration gas must be corrected to standard conditions (0 C 760 mm Hg and dry) Needless to say that a careful calibration of the gas meter or flow rator is of the same importance as it is for the gas analyser

The calculation of gas exchange would be simplified and shortened if the diaferometer could be calibrated directly in metabolic O_2-deficit instead of the true deficit in such a way that the span should be adjusted to an E M.F (O_2) corresponding with 320 mV instead of 254 8 mV as in the above mentioned example of calibration

The ratio metabolic O_2-deficit to true deficit is therefore $\frac{320}{254\ 8} = 1\ 25$ and it can easily be proved that this ratio is a constant, quite independent of the CO_2 production and O_2-consumption of the living subject (Table I)

TABLE I

True O_2-deficit and Metabolic O_2-deficit at different levels of O_2-consumption Ventilation rate = 100 l/min

O_2-cons. l/min	O_2-content of CO_2-free gas	True O_2-deficit	Metabolic O_2-deficit	Ratio MD/TD
0 20	20 78	0 16	0 20	1 25
0.50	20 54	0 40	0 50	1 25
1 00	20 14	0 80	1 00	1 25
2 00	19 33	1 61	2.00	1 25

From Table I it becomes clear that a calibration of the O_2-diaferometer in metabolic O_2-deficit is completely justified and in this new method of calibration the number of millivolts indicated by the recorder should be 25 per Cent higher than the figure

corresponding to the "true" difference in O_2-percentage of the gases in the reference and detection channels of the diaferometer

The E.M.F.$_{(O_2)}$ in the second calibration is therefore 1.25 × E.M.F.$_{(O_2)}$ of the first calibration Both types of calibration can therefore easily be compared.

For calculation of total CO_2-production the "metabolic CO_2-surplus" may be considered equal to the "true CO_2-surplus" on account of the very low CO_2-percentage of fresh outdoor air

The formulas (3) and (4) should undergo a slight modification for adapting them to the new calibration

$$\text{Metabolic } CO_2\text{-surplus} = \text{E.M.F.}_{(CO_2)} - \frac{0.12}{1.2_5}\ \text{E.M.F.}_{(O_2)}$$

$$\text{Metabolic } CO_2\text{-surplus} = \text{E.M.F.}_{(CO_2)} - 0.1\ \ \text{E.M.F.}_{(O_2)} \qquad (5)$$

$$\text{Metabolic } O_2\text{-deficit} = \text{E.M.F.}_{(O_2)}. \qquad (6)$$

The "metabolic CO_2-surplus" and the "metabolic O_2-deficit" are therefore fictive percentages of these gases by which the total flow (V l/min) should be multiplied to find directly the total CO_2-production and the O_2-consumption of the subject under investigation.

A correction for R.Q is no longer necessary and the calculation of results is therefore considerably facilitated.

During the metabolic experiment the deflection of the recorder gives immediately a quantitative picture of the O_2-consumption (V remaining constant) and the figure for the R.Q can be quickly calculated from both records at any time of the experiment

The introduction of the "span" at the one side and the "metabolic gas percentage" on the other has therefore considerable advantages over the procedures commonly used in metabolic research with the diaferometer

4. CONCLUSION AND FINAL SUGGESTIONS

Both methods of calibration of the diaferometer have their advantages and disadvantages the first method makes the apparatus a gas analyser though not with absolute specificity the second method modifies the diaferometer to a respirometer at least in combination with a suitable gas meter or flow rator

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The calculation of gas exchange would be simplified and shortened if the diaferometer could be calibrated directly in metabolic O_2-deficit instead of the true deficit in such a way that the span should be adjusted to an E.M F (O_2) corresponding with 320 mV instead of 254 8 mV as in the above mentioned example of calibration.

The ratio metabolic O_2-deficit to true deficit is therefore $\frac{320}{254\ 8} = 1\ 25$ and it can easily be proved that this ratio is a constant, *quite independent of the CO_2-production and O_2-consumption* of the living subject (Table I)

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O_2-cons. l/min	O_2-content of CO_2-free gas	True O_2-deficit	Metabolic O_2-deficit	Ratio MD/TD
0.20	20.78	0 16	0 20	1 25
0 50	20.54	0 40	0.50	1.25
1 00	20.14	0 80	1 00	1 25
2 00	19 33	1 61	2.00	1 25

From Table I it becomes clear that a calibration of the O_2-diaferometer in metabolic O_2-deficit is completely justified and in this new method of calibration the number *of millivolts indicated* by the recorder should be 25 per Cent higher than the figure

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The formulas (3) and (4) should undergo a slight modification for adapting them to the new calibration:

$$\text{Metabolic } CO_2\text{-surplus} = \text{E.M.F.}_{(CO_2)} - \frac{0.1}{1.25}\ \text{E.M.F.}_{(O_2)}.$$

$$\text{Metabolic } CO_2\text{-surplus} = \text{E.M.F.}_{(CO_2)} - 0.1\ \ \text{E.M.F.}_{(O_2)} \qquad (5)$$

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0 50	20 54	0 40	0 50	1.25
1 00	20 14	0.80	1 00	1 25
2.00	19 33	1 61	2.00	1 25

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subjects, and does not contain traces of alcohol volatile fatty acids or acetone. The expiration air of ruminants is not an adaequate calibration gas on account of its content of methane or even hydrogen.

SUMMARY

Some improvements of gas analysis in metabolic experiments have been described, more particularly in using methods, based on heat conductivity of the respiratory gases (diaferometer)

The calculation of the total gas exchange of the living subject could be shortened and simplified by calibrating the gas analyser as a respirometer introducing the term "Metabolic Oxygen deficit" and "Metabolic Carbon dioxide surplus"

REFERENCES

Romijn C. and W. Lokhorst Acta Physiol. Pharmacol. Neerlandica 8; 137 151 (1959).

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Of course both methods make the apparatus suitable for continuous recording of the gas exchange of a living subject particularly in long term experiments in case of calibration as a gas analyser the calculation of results is more laborious than it is after the second calibration as a respirometer The diaferometer however has been introduced originally as an apparatus for determining respiratory metabolism in man and animals we prefer therefore the appropriate calibration introducing the terms Metabolic O_2-deficit and Metabolic CO_2-surplus

For convenience of research workers in laboratory or clinic we would propose the following procedure of accurate calibration or recalibration

1 Collect an amount of expiration gas in a rubber bag
2 Dilute the gas with air till the CO_2-content of the mixture will be 0 8 per Cent approximately
3 After thoroughly mixing a sample should be analysed chemically in a gas analyser of the Haldane type
4 Suppose the gas composition is 0 84 % CO_2 19 94 % O_2 and 79 22 % N_2
5 The metabolic CO_2-surplus is 0 84 – 0 04 = 0 80 %
The metabolic O_2-deficit is

$$1\ 25\left(20\ 94 - \frac{100 \times \%O_2}{100 - \%CO_2}\right) = 1\ 25\left(20\ 94 - \frac{1994}{99\ 16}\right) = 1\ 04\ \%$$

6 The gas mixture should be presented to the diaferometer after running the instrument with outdoor air to adjust the zero recording
7 Adjust the span of the O_2-diaferometer in order to fix the recorder deflection on 104 mV (6)
8 *Adjust the span* of the CO_2-diaferometer in order to fix the deflection of the recorder on $80 + 0\ 1 \times 104 = 90\ 4$ mV (5)

It should be emphasized that bomb gases must never be used in calibration of a diaferometer on account of their relatively high content of inert gases with heat conductivities considerably divergent from that of air Expiration gas is the most *physiological* medium for calibration provided it has been taken from healthy

subjects, and does not contain traces of alcohol, volatile fatty acids or acetone The expiration air of ruminants is not an adaequate calibration gas on account of its content of methane or even hydrogen.

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MOTILITY CYCLES IN THE STOMACH OF THE FASTING DOG

BY

J R. ROBORGH

In recent years some radio telemetering devices were developed to register changes in pressure in the animal body (FARRAR and BERNSTEIN 1958 FARRAR BERKLEY and ZWORYKIN 1960 CONNELL and ROWLANDS 1960 SMYTH and WOLFF 1960 SMITH and RIDGWAY 1961 1962 MACKAY 1961 WATSON ROSS and KAY 1962 ROSS WATSON and KAY 1963) In order to investigate the potentialities of this new method we performed a short series of experiments on dogs

1 METHOD

The apparatus used consists of a 23 × 9 mm capsule containing a pressure-sensitive transducer and a radio emitter as described by SMITH and RIDGWAY (1961) and manufactured by Solartron Ltd. The radio signals are detected by a receiver and permanent recordings of the pressure changes in the animal body are registered by a strip chart recorder Before each experiment the capsule is calibrated by submersion at different depths in water at body temperature. Thus a calibration trace of the responses to various pressures is obtained

When testing the method we restricted our experiments to the stomach Therefore we anchored the capsule–also called (endo-) radio probe or radiopill–by means of a nylon thread (0 3 mm in diameter) which was cemented centrally to the battery end of the capsule

2. HANDLING OF THE DOGS

Conscious light weight (9–11 kg) crossbred female dogs were used. As the radio probe is very sensitive it is necessary that during the experimental period about 6 to 7 hours in our cases

the dogs are lying fully relaxed. It goes without saying that it takes rather long training to accustom them to do so Four dogs were tried, two of which, aged half a year and three years respectively proved to be co-operative to an astonishing degree

In all experiments reported here the dogs were fed till 5 p.m. of the day preceding the recording day On the morning of the recording day the dog was injected intravenously with 0.2 ml of a preanesthetic containing only 2 mg of acetylpromazine bimaleate. About 10 minutes later the dog was lightly narcotized with ether In the meantime the nylon thread of the radio probe was drawn through the lumen of a thick-walled plastic tube with an outer diameter of 6 mm by means of a bodkin Then it was possible to push the tube plus radio probe down into the stomach of the dog by pulling slightly at the free end of the thread, thus straightening the tube and the erected probe at its foremost end. When the tube was afterwards pulled back the probe sometimes followed the tube a little upwards but this caused no trouble at all as the dog swallowed the probe further down into its stomach as soon as it recovered from the narcosis. The nylon thread was led via the corner of the mouth and tightly fastened to the collar Of course the length of the thread is critical for each dog We have reason to believe that during the recordings the radio probe remained in the pars pylorica When the probe was properly placed and anchored the now conscious dog was laid down on its *right* side in a tilted wooden box (measuring 63 × 43 × 41 cm) placed on a table in such a way that the dog was easily accessible

Three aerial loops surrounding the box lengthwise breadthwise and depthwise were connected with the complex radio receiver by means of a switch. In practice one and the same aerial could be used during the recordings, but sometimes one of the two other aerials gave a stronger signal

3. RESULTS

Probably due to the preanesthetic and the ether narcosis the recording showed less specific tracings during the first two hours. After that time however typical motility cycles appeared, consisting of sharp-cut periods of almost complete rest during about 1½ hours alternating with periods of strong contractions of about ~0 minutes.

The recordings of four representative sessions, covering 1233 minutes of observation of the same dog under identical conditions showed a total of 220 minutes of contractions, or 10 per Cent of the total recording time (see table for details)

TABLE 1

Time relation between rest and contraction periods

recording date	observation time	periods of rest (min)	periods of contractions (min)
March 26, 1964	10 –5 a.m	91	18
	to 4 15 p.m.	10*	1
		104	18
April 15 1964	11 13 a.m.	105	23
	to 3 36 p.m	118	17
April 27 1964	10 38 a.m	0	20
	to 4 18 p.m.	103	23
		91	–7
June 30 1964	11 20 a.m.	131	*0
	to 4 p.m	0	37
mean value:		100	23
standard error of the mean:		± 5 4	± 2.1

During the quiescent periods the stomach showed practically no movements at all or only slight and regular ones. The respiration proved often to be the only cause of deviations from the base line on the recording paper corresponding to only about one cm of water pressure.

In the course of a contraction period however some tens of pressure excursions occurred indicating pressures of 60 to 90 cm of water and sometimes surpassing the 100 cm level It is interesting that although the dog was dozing or sleeping most of the time in its box it never woke up or reacted at the sudden onset of the contractions nor during those periods

Our experience with the other dog was of exactly the same nature in all respects

4. DISCUSSION

It was *surprising* to learn that in recent literature data concerning the cyclic nature of the motility of the stomach in the

fasting state proved to be rather scarce. Going back in the literature as far as half a century ago however we encountered a magnificent and elaborate study on the gastric motility of the fasting dog by BOLDYREFF (1905, 1911) a pupil of PAVLOV This author studied the motility and the secretion of the stomach and duodenum in sometimes plurally fistulated dogs in which a balloon connected to a manometer had been inserted via a fistula. He stated that the stomach shows intermittent contraction periods after the di gestion of the last meal. The contraction periods, lasting 20 to 30 minutes, alternated with periods of rest of about two hours. This cyclic behaviour could be maintained several days, after which it became more irregular and fainter

It is clear that our observations are in fair accordance with the well-documented experiences of BOLDYREFF although distinctly different methods were used.

Gastric motility cycles of the empty stomach of some other animals have been reported. MORRISON LIN ECKEL, VAN ITALLIE and MAYER (1958) described a cyclic pattern with a periodicity of 15 to 20 minutes in rats. SHARMA ANAND DUA and SINGH (1961) working with cats and monkeys mentioned contraction periods of 20 to 30 minutes alternating with quiescent intervals of 15 to 20 minutes.

The *bursts of contractions of the empty human stomach* are known as hunger contractions and there are considerable differ ences of opinion concerning the correlation between these contractions and the feeling of hunger pangs (HOELZEL, 1960) PENICK, SMITH, WIENEKE and HINKLE (1963) studied this matter in healthy young men, using three different methods, including the pressure sensitive radio probe. They found no relation between the intensity of hunger reported and the frequency of gastric contractions, whilst their findings strongly suggest that the gastric balloon can activate gastric contractions. The latter conclusion is in accordance with the experiences of HOELZEL. The use of a balloon is also criticized by other authors because it could give rise to so-called *tetanus* of the stomach. JAMES (1957) stated that tetanus of the stomach is probably an artifact.

In connexion with the objections against the balloon method mentioned above we would point to the remarkable agreement of

our observations with the results of BOLDYREFF who used the balloon method.

With regard to the results of PENICK SMITH WIENEKE and HINKLE it may be doubted that they observed real hunger contractions as they stated that these contractions occurred only singly or occasionally in groups of two or three and that their subjects fasted for only 5 hours. In the two cases of the 20-hour fasts of two subjects they may easily have missed the bursts of contractions because of the relatively short time of observation (2 hours) for FARRAR and BERNSTEIN (1958) observed long periods of complete quiescence sometimes lasting for more than two hours.

The radio probe may be considered as a valuable tool for the study of gastro-intestinal physiology A serious drawback however is the relatively short life of the battery (about 30 hours) the more so as reviving of the probe by replacement of the used battery proved to be a delicate job

5 SUMMARY

The motility pattern of the stomach of the fasting dog was studied by means of a radio probe The stomach of the fasting dog exhibits regular periods of strong contractions lasting about *0 minutes, which alternate with periods of almost complete rest during about 1½ hours.

The observations resemble closely the results of an extensive study of BOLDYREFF (1905 1911) who applied the balloon method per fistulam. The strong similarity of the results obtained by the two different methods is stressed.

As far as we know this is the first report on the motility pattern of the empty stomach of dogs recorded by the radio probe method.

ACKNOWLEDGEMENT

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Besides being primarily determined by factors such as intensity duration, and spatial organization of the stimuli, the responses are known to be strongly influenced by "central factors such as alertness and attention level arousal and interaction between different sensory channels, and in the last few years several investigators and groups of investigators have studied these influences, thus introducing even more variables which, moreover do not easily lend themselves to quantitative treatment if, indeed, they can be defined at all. In view of this it is hardly a matter for surprise that, here again, widely differing opinions are held and conflicting conclusions have been drawn notably as regards the influence of attentiveness' on the amplitude of some more or less arbitrarily chosen element or elements of the response

In the experiments in question tasks of various types have been used to focus the subjects attention on the stimuli or to draw it away from them.

Flash-counting tasks were given by JOUVET (1957), VAN HOF *et al* (196.) EASON (1964) GUERRERO-FIGUEROA (1964) other tasks designed to keep the subjects attention focused on the stimuli include vigilance tasks where the subject is to respond by pressing a key to slightly dimmer signal flashes randomly interspersed between standard flashes (HAIDER c.s. 1964 SPONG c.s 1965)

"Distractive" tasks include performing mental calculations (JOUVET l.c. GARCÍA-AUSTT 1964) and focusing the attention on stimuli of a different modality offered isoperiodically with the flashes (GUERRERO-FIGUEROA, l.c., GARCÍA-AUSTT l.c.) A different type of task is given by SUTTON (1964) the subject is to guess what the modality (visual or auditory) of a test stimulus given, at a fixed interval, after a cueing stimulus is going to be.

Most authors (Jouvet, Guerrero-Figueroa, García-Austt, Spong c.s. Sutton) report an increase in amplitude of the VER when the subjects attention is focused on the visual stimuli. It is, however not always easy to find out to what response element they refer Jouvet Guerrero-Figueroa, and García-Austt also state that amplitude decreases when the subject's attention is focused elsewhere Van Hof *et al.* on the other hand, find no effect of flash counting in 14 out of 15 subjects, while according to EASON (l.c.) "any stimulus situation such as counting the stimuli but also including such things as thinking of weight lifting, increases the

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INFLUENCE OF ATTENTIVENESS OF VIGILANCE TASK DIFFICULTY AND OF HABITUATION ON CORTICAL EVOKED RESPONSES AND ON ARTIFACTS

BY

W J RIETVELD W E M TORDOIR AND J R B HAGENOUW

CONTENTS

1 INTRODUCTION

In a previous paper (RIETVELD 1963) it was argued that the differences between descriptions in the literature of the occipito-cortical response to light flashes (visual evoked response VER) are largely due to differences in experimental set up (flash duration flash intensity interval between flashes position of electrodes etc.) resulting among other things in different degrees of contamination of the response by artifacts

subdural electrodes. The question naturally arises whether the reported increase in amplitude when the subject's attention is fixed upon the stimuli might not partly or wholly be due to an increase in artifact amplitude [1])

The present report falls into two parts. The first is mainly a report on two parallel series of experiments. In those of the first series some experiments made by Spong *et al.* are faithfully reproduced in those of the second, they are modified to minimize artifacts and to ensure uniformity of response [2])

In the experiments of Spong et al. alternating clicks and flashes are offered at one-second stimulus intervals, so the frequency of either is one per two seconds. Three types of tasks are used, viz. visual vigilance task (instructions ignore clicks, do not respond to standard flashes, press key on dimmer signal flashes) and its auditory counterpart, a key pressing task (press key in response to every click or flash) and counting task (count each stimulus in the modality t which attending; press key after 50 stimuli have been counted). In the experiments t be described the vigilance tasks only have been retained.

Besides showing that the difference between the results obtained by Van Hof *et al.* and by Sutton and those reported by the other authors named cannot be explained on the basis of absence of artifact in the first-named experiments, the results obtained in the present experiments suggested that other factors, viz. the difficulty of the task and the degree of habituation of the subject to the experimental situation might be involved. This led to a third series of experiments in which the influence of these factors was studied

2. INFLUENCE OF ATTENTION ON CORTICAL EVOKED RESPONSE AND ON ARTIFACT

.1 Methods

All experiments are made in a dark, soundproof room. Unipolar leads are used throughout the active occipital electrode lies at

[1]) Of course such an explanation would not account for the increase, in the case of attention of responses led off from subcortical structures as reported by Jouvet.

[2]) The experiments of Spong *et al.* were chosen because they are clearly and comprehensibly described, so that they could be imitated *faithfully*

amplitude of the VER over that in the 'blank mind' situation.

In the hands of Spong e.a. stimulus counting gave inconsistent results. They argue that counting stimuli may be distracting rather than focusing attention on the stimuli because of the necessity of keeping track of the number counted

Some results have been reported for other stimulus modalities Thus DAVIS (1964) in an experimental situation somewhat analogous to that of Haider *et al* finds that the uncertainty of whether a given tone pip in a cycle will be somewhat louder or weaker than those preceding and following it increases the response amplitude. Davis specifically refers to slow waves.

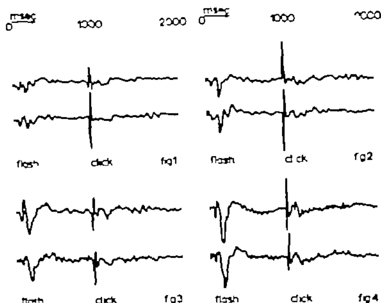
SATTERFIELD and CHEATUM (1964) report that for shock stimuli delivered at the wrist the response amplitude is reduced in the case of attentiveness and increased in that of nonattention Later however SATTERFIELD (1965) reports that both for shocks and for clicks attentiveness results in an increase in amplitude in 41 (40) out of 47 subjects and states that the decrease reported earlier was found in 5 of 25 subjects only What happened in the other cases is not clear

The importance of the uncertainty factor (DAVIS l c) was stressed by Sutton Uncertainty as to the modality of an expected stimulus increases the amplitude of the response the increase being more marked as the guess proves to be wrong

In the paper referred to earlier (RIETVELD l c.) it was reported that the recorded response can be considered as free from artifact if the flash frequency is not below 3/sec and if unipolar leads are used with the active electrode at no more than about 2 cm above the inion

Among further precautions to ensure uniformity of response are maximal dilation of the pupil and prevention of fixation difficulties The first condition (flash frequency > 3/sec) is fulfilled only in the experiments by Van Hof *et al* (3/sec) who found no effect of flash counting and in those by Sutton (5/sec) who found no specific effect the second (active electrode about 2 cm above inion) in those by Van Hof *et al* by Sutton by Haider Spong Lindsley [1]) and by Guerrero-Figueroa who for that matter used

[1]) In the experiments made by these investigators, the electrode lies about 2 cm above the inion and about _ cm to the side of the midline. This, however makes no difference



Figs. 1–4

In Fig 1 the flash response is not very marked there is a brief fast response starting about 30 msec after the click, which from its timing and its shape can be identified with BICKFORD s (1964) muscle artifact as is further proved by its behaviour when the skin over the scalp is stretched. When the attention is focused on the auditory stimuli the occipital flash response shows a late (about 170 msec) deep surface-positive trough the fast click response is very large. With the attention centered on the flashes the VER is more marked in the temporal lead than in the occipital one it is again dominated by a deep late surface-positive wave which can, from its shape and its time relations, be identified with that in Fig Again, the early fast click response is present it is followed by one or two not very marked, slower waves, the main feature being a rather broad surface-negative wave at some 1"0 msec

When the attention is focused on the clicks the surface-positive wave in the temporal flash response is far deeper than in any of the other cases the fast click response is again present and the slower response is more marked than in Fig 3

about 2 cm above the inion the temporal electrode in the frontal plane through the external meati at 8 cm from the midline the reference electrode is at the right earlobe Responses are averaged with the aid of a Nuclear Data Enhancetron computer

Series 1 The observer s eyes are open Standard stimuli are GR Strobotac flashes delivered on an 10×10 cm² opal glass window at 30 cm from the observer (screen luminance about 950 mL) and 80 dB clicks duration 5 msec. Signal flashes have an intensity of about 25 pCt of standard flash intensity signal clicks are 60 dB Stimuli are offered in series of 330 30 of which are test stimuli. The interval between successive stimuli is one second signal stimuli are randomly mixed among the standard stimuli only the responses to the standard flashes and clicks are averaged

Series 2 The experiments in this series differ from those of Series 1 in the following

- The visual vigilance task only is retained
- The subject s pupils are maximally dilated by means of instillation of 10 pCt phenylephrine-HCl in the conjunctival sacs
- A piece of plain white paper is placed before the subject s eyes to prevent fixation difficulties
- Screen luminance of standard flashes is reduced to 500 mL to prevent saturation effects in the response
- Flashes are offered either at 1/sec or 3/sec no clicks are presented
- To distract attention from the flashes an auditory task is given the subject is instructed to listen to and to translate a spoken text to which noise has been added
- A supraorbital electrode is added to detect any eyeball and eyelid artifacts.

Nine subjects were used in Series 1 they were among the eleven used in Series 2

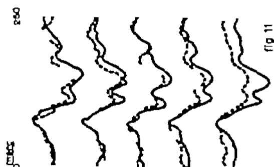
2 2 Results

2 2 1 *Series 1*

The averaged responses obtained in one subject and in four sets of two series of stimuli are shown in Figs. 1–4

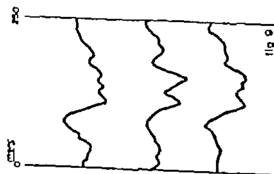
Fig 1 shows the occipital responses to flashes and to clicks when attention is focused on the visual stimuli the occipital response when attention is centered on the clicks is shown in Fig 2 Figs. 3 and 4 give the corresponding temporal responses.

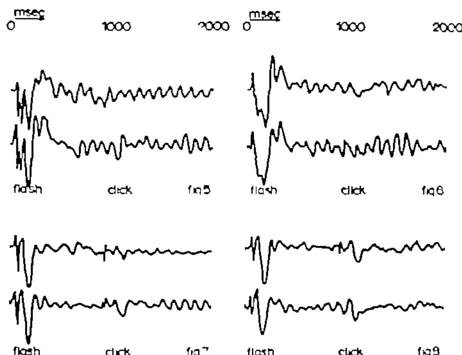
Figs. 10 and 11 show respectively the averaged responses obtained during distraction and during attention (broken lines) together with those found in the no-task situation. In all cases the peaks of the early surface-negative component tentatively identified as the B-wave were made to coincide.





Figs. 9–11





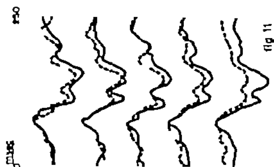
Figs. 5–8

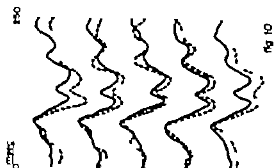
The corresponding responses in another of the subjects are pictured in Figs. 5–8. Here in all cases the flash response in which the Γ trough can be tentatively identified is dominated by a deep late (about 185 msec) surface-positive trough. The peak-to-peak B Γ (?) amplitude is greater when attention is centered on the visual stimuli. Click responses are far less marked than in the first subject: in Figs. 5 and 6 (occipital leads) no click response can be seen; the early fast response can be recognized in the temporal leads while the late slow response elements which are of small amplitude in Fig. 7 are somewhat larger in Fig. 8, that is when attention is focused on the clicks.

2.2.2 *Series 2*

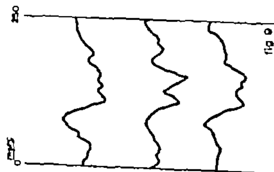
Typical results obtained in the experiments of Series 2 are exemplified by Figs. 9–11. Fig. 9 shows the results of averaging over three series of responses (flash frequency 3/sec) when no task had been allotted to the subject. It will be seen that, as found earlier, time relations are more constant than amplitude relations but that the latter are not too far different.

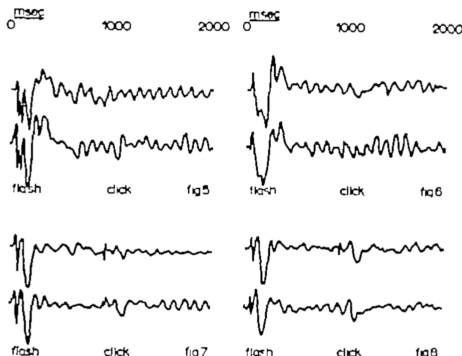
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It is clear that in all cases the amplitude of the uncontaminated response increases upon distraction and decreases upon attention.

2.2.3. *Discussion effect of attention on artifact size*

The results obtained in the experiments so far described show that the contaminated visual evoked response (Series 1) as well as the uncontaminated response of Series 2 decreases in amplitude when attention is focused on the visual stimuli, so it appears that an artifact increase cannot very well be invoked in explanation of the fact that most authors describe an increase instead of the decrease found in the present experiments.

That, under the conditions of the experiment, the eyeball artifact actually diminishes in size when attention is focused on the visual stimuli is apparent from Figs. 12–14 which show records obtained, under conditions as in Series 1 from occipital and supraorbital leads.

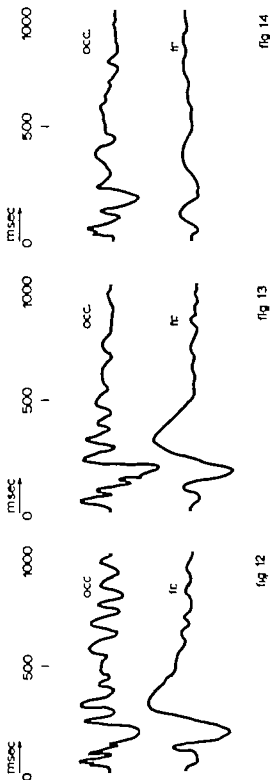
The averaged responses in the no-task, no-distraction situation (absence of auditory stimulation) are shown in Fig 12. The occipital response shows in this case again late deep, surface-positive wave which was conspicuously beent in the uncontaminated responses of Series 2. The supraorbital response shows corresponding trough which is followed by a surface-negative wave at about 350 msec. The introduction of auditory stimulation (Fig. 13) does not seem to make much difference

When, however the attention is focused on the flashes the amplitude of the trough in the supraorbital response is reduced to nearly zero, and the corresponding wave in the occipital lead is correspondingly diminished (Fig. 14) That the wave in question originates at the eyeball, so that the late surface-positive wave found in the experiments of Series 1 can be identified as an artifact, is apparent from Fig. 15 which gives the responses from the infraorbital and supraorbital leads (not inversion of polarity) and from leads 4 3, 2, and 1

3 INFLUENCE OF TASK DIFFICULTY AND OF HABITUATION ON RESPONSE AMPLITUDE

3 1 Considerations

There is a marked discrepancy between the visual and the auditory evoked responses as regards the influence of attention. While, as mentioned above focusing of the attention on the visual stimuli caused a decrease in amplitude both of the true VER and of the artifacts, if present attentiveness directed towards the auditory stimuli results in an increase in amplitude of the auditory evoked response itself as well as of the Bickford artifact. Such an



Figs. 12–14

difference between the visual and auditory systems. This in its turn, suggests experiments where the effect of attention is studied when the visual task is made easier and the auditory task more difficult.

3. EFFECT OF ATTENTION AT VARIOUS DEGREES OF DIFFICULTY OF VISUAL TASK EFFECT OF HABITUATION

The visual task is experienced as being less difficult and less disagreeable when screen luminance is lowered, the difference between standard flashes and signal flashes being kept at 25 pCt. of standard flash luminance

Fig 16 shows the occipital responses at standard flash luminances of 500 50 and 5 mL at flash frequencies of 1/sec 3/sec, and 5/sec with and without attention, as recorded from one member of a fresh group of inexperienced subjects.

At 3/sec, when the task is still experienced as being difficult at 50 mL, the amplitude is, both at 500 and 50 mL, smaller in the case of attention at the lowest luminance there is no difference At 1/sec and at the highest luminance attention still results in a decrease in response amplitude at 50 and at 5 mL, however it results in an increase The same applies to 5/sec stimulation.

Of the other nine members of this group seven behaved similarly in the other two there was no consistent pattern.

Of course the 1/sec responses are contaminated by artifact as is clearly shown in Fig 17 which gives the responses of another of the ten subjects for occipital and frontal leads. At low luminance, and with attention focused on the visual stimuli, the artifact is negligible, so the deepening f the Γ trough is real. The figure also shows the fragmentation of B at high luminance described earlier t be more marked in the case of attention

The effect of habituation was studied in two members of this group

Figs. 18 and 19 show the results obtained in one of them in five pairs of series (screen luminance of standard flashes, 500 mL flash frequency 1/sec occipital lead) when the subject had never before experienced the experimental situation, and in three pairs run under the same conditions one month and 5 experimental sessions later It is clear that in the first case attention results in a decrease and in the second in an increase of response amplitude

opposite behaviour of two sensory channels is of course quite unexpected and suggests effects of factors not studied so far

The subjects were unanimous in declaring that the visual task was much more difficult than the auditory task that at 3 flashes

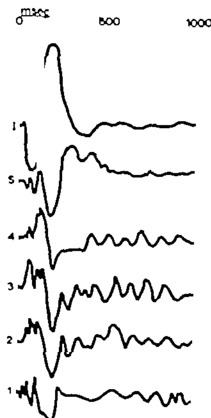
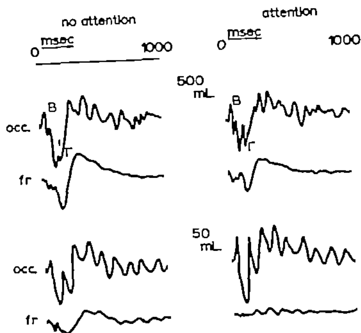


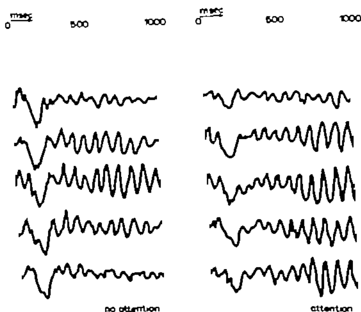
Fig 15

per second in particular was experienced as being extremely difficult and unpleasant far more so than at either 1/sec or 5/sec. Also it was reported that after several series had been run the task became less difficult Parallel to this there was a marked difference in performance In the auditory task the signal clicks were correctly detected without either false-positives or false-negatives. In the visual task however there was a notable proportion (up to 10 pCt.) of false positives. With experience performance appeared to improve All this suggests that the different behaviour as regards the influence of attention of the channels might be related to task difficulty rather than to a fundamental



no attention
attention
0 msec
1000
0 msec
1000
500 mL
B
occ.
Γ
fr
B
Γ
50 mL
occ.
fr

Fig 17



0 msec
500
1000
0 msec
500
1000
no attention
attention

Fig 18.

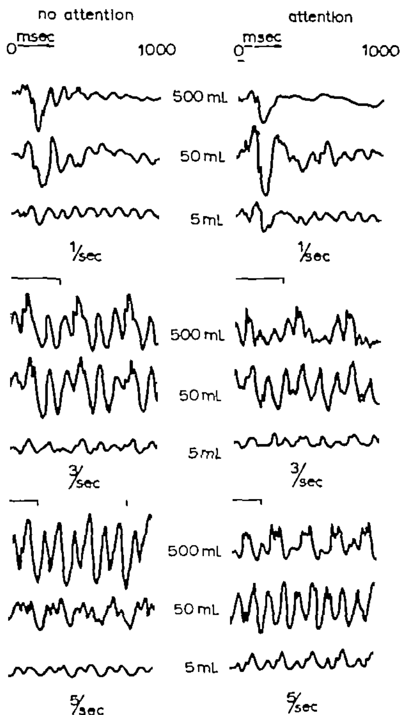

no attention
attention
0 msec 1000
0 msec 1000
500 mL
50 mL
5 mL
1/sec
1/sec
500 mL
50 mL
5 mL
3/sec
3/sec
500 mL
50 mL
5 mL
5/sec
5/sec

Fig 10

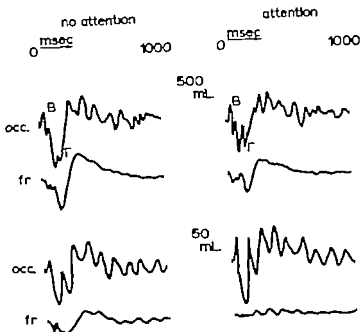

no attention
attention
0 msec
1000
0 msec
1000
500 mL
B
Γ
occ.
fr
B
Γ
50 mL
occ.
fr

Fig 17

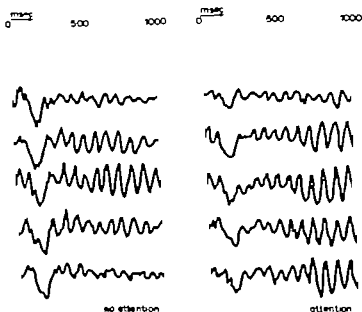

0 msec
500
1000
0 msec
500
1000
no attention
attention

Fig. 18.

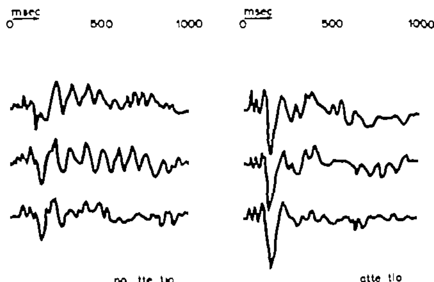


Fig 19

3 3 EFFECT OF ATTENTION AT VARYING DEGREES OF DIFFICULTY OF AUDITORY TASK

The auditory task is experienced as being more difficult when the intensity of the standard clicks is reduced from 80 to 15 dB the intensity ratio between standard and signal clicks remaining the same Fig 20 shows click responses (temporal lead) at the two intensities with and without attention While as reported in section 2 2 1 attention to the clicks results in an increase in response amplitude at the higher intensity it causes a response diminution at the lower one

4 COMMENT BEHAVIOUR OF SPONTANEOUS" BACKGROUND EEG

Two factors are involved in the influence of attentiveness on the cortical evoked response viz the effect on sensory inflow

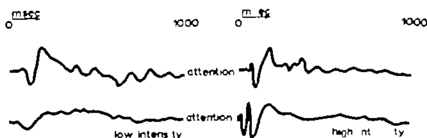


Fig 20

i.e. on the amplitude of responses led off from subcortical stages of the sensory channel and that on cortical responsiveness.

JOUVET (l.c.) found that the response to light flashes led off from the optic radiation increases in amplitude when attention is focused on the stimuli in a flash-counting task on the other hand, HERNÁNDEZ-PEÓN *et al.* (1957) state that the amplitude of the response recorded with the aid of subcortically implanted electrodes diminishes when a distracting task (mental calculation) is given. This would suggest that the decrease in amplitude of the cortical response in the case of a difficult task is due to a decrease in cortical responsiveness. That there is indeed a difference in cortical behaviour at high flash intensity and that at lower intensity is apparent from the background EEG s shown in Figs. 21 to 24 which were recorded, with the aid of a Grass polygraph, during the experiment to which Fig 17 refers.

In the upper tracks the moments at which the flashes are presented and the reactions of the subject to (supposed) signal flashes are indicated (arrows) the lower tracks show the EEG as recorded from an electrode lying in the midline at six cm before the inion ("lead 3").

To obtain a rough estimate of α-activity lines were drawn parallel to the zero line at $+1$ cm and -1 cm, and the number of positive and negative peaks exceeding these levels was counted over a 12 sec period.

The "α-index thus obtained is highest (78) at the lower luminance (50 mL) when attention is not directed to the flashes as expected, it is markedly lower (26) when it is focused on them. At 500 mL, and in the non-attention situation it is somewhat lower than at 50 mL (68 against 8) with the attention directed to the flashes it drops to 2 indicating a strong suppression of the α activity (desynchronisation).

The habituation effect is also reflected in the background EEG as shown in Figs. 25 to 28 The records in question were made as part of the experiment to which Figs. 18 and 19 refer

Fig 25 shows the EEG of the unexperienced subject in the non-attention situation after he has become used to the procedure α-activity during the experimental session is much higher (Fig 26)

Figs. 27 and 28 show the corresponding EEG s when attention is centered on the stimuli though the difference is less since there

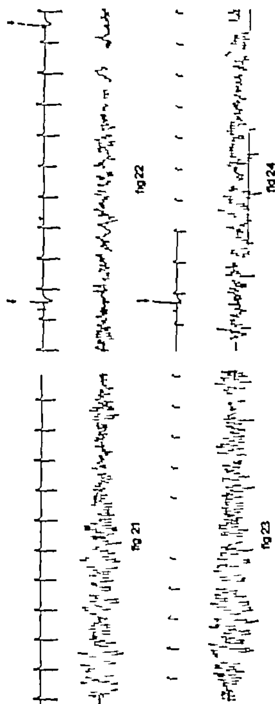


Fig. 21 Background EEG; high luminance no attention. Fig. 22. Background EEG high luminance attention. Fig. 23. Background EEG; low luminance, no attention. Fig. 24 Background EEG; low luminance attention.

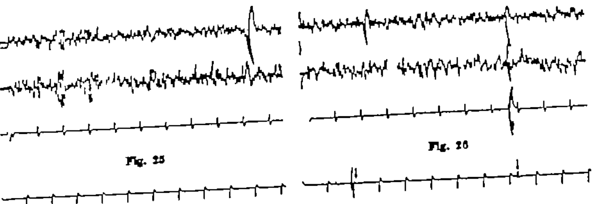


Fig. 25

Fig. 27

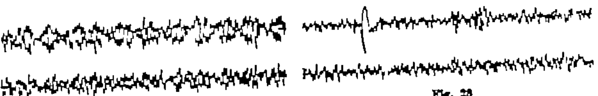


Fig. 26

Fig. 28

Fig. 25. Background EEG; subject inexperienced no attention. Fig. 26. Background EEG; subject experienced, no attention. Fig 27 Background EEG; subject inexperienced, ttention.

Fig. 28. Background EEG; subject experienced, attention.

Upper tracings active electrode 2½ cm above the inion; lower tracings 2½ cm above the inion and 2½ cm from the midline

still is, in the experienced subject, a marked suppression of α activity; the trend towards diminution of α suppression during performance of the task is evident.

SUMMARY

Nearly all authors describe an increase in amplitude of cortical evoked responses both to visual and auditory stimulation when the subject's attention is focused on the stimuli. From the descriptions of the experiments where the visual evoked response was investigated it can be inferred that in most cases, the recorded responses were contaminated by artifacts. Since the two papers in which no such increase was reported are the only ones where the recorded response could be supposed to be free from artifacts, the question naturally arose whether, in those cases, the observed increase *might not be due to an increase of artifact amplitude*. Two series of experiments where visual and auditory vigilance tasks were given, in one case under conditions when the response was contaminated and in the other where contamination was excluded, showed that the auditory channel appeared to behave differently from the visual one in that, while in the case of visual stimulation attention caused a decrease in amplitude both of the uncontaminated response and of the artifacts—so that an artifact increase cannot be invoked in the explanation of the response increase reported by most authors—attention increased the amplitude of the response and of the artifact in the case of auditory stimulation. The visual task was experienced as much more difficult than the auditory task; in the latter performance was much better. The visual task appeared to become somewhat easier with habituation, and performance improved.

In a third group of experiments the visual task was made easier and the auditory task more difficult. Now attentiveness increased response amplitude in the first case and diminished it in the second. This, together with the fact that subcortical responses have been reported to increase *in the attentive state, suggests that in the case of a strong stimulation* and of a difficult task cortical responsiveness is lowered. Background EEG's show that, under these conditions, there is a strong suppression of the alpha-rhythm; this suppression becomes less marked as the subject gets used to the procedure.

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NETHERLANDS SOCIETY FOR PHYSIOLOGY AND PHARMACOLOGY

PROCEEDINGS SIXTH FEDERATIVE MEETING OF MEDICAL-BIOLOGICAL SOCIETIES LEIDEN JUNE 10–11 1965

PROGRAMME

P N Aarson *The influence of analgesic antipyretic drugs on the responses of guinea-pig lungs to bradykinin*

Department of Pharmacology University of Amsterdam

Bradykinin increases the resistance to inflation of guinea pig lungs *in vivo* which effect is specifically antagonized by antipyretic analgesic drugs (Collier, Holgate Schachter and Shorley 1959 1960 Collier and Shorley 1960)

If these drugs are acting on specific receptors for bradykinin in the bronchial muscles as suggested by Collier (1962) they must also counteract the bronchoconstriction caused by bradykinin in the isolated guinea pig lung

In isolated guinea pig lung preparations according to the methods described by Sollmann and von Oettingen (1928) and by Bhattacharya and Delaunois (1955) relatively high doses of bradykinin were needed to cause bronchoconstriction which effect was accompanied by an obvious constriction of the perfused lung vessels. These responses, however were not reduced by calcium acetylsalicylate sodium salicylate sodium phenylbutazone amidopyrine and phenazone in concentrations of 40 μg per ml perfusion fluid.

Since these findings were in contrast to the effects *in vivo* described by Collier and Shorley (1960) the *in vivo* system was re-investigated The mean intravenous threshold dose of bradykinin producing an increase in resistance to inflation of guinea pig lungs *in vivo* was 0 6 μg per kg body weight and thus, in spite of the rapid destruction of bradykinin in blood many times smaller than the dose of bradykinin needed to produce bronchoconstriction in isolated lungs The increase in resistance to inflation of lungs due to an intravenous threshold dose of bradykinin coincided with a secondary rise in systemic arterial blood pressure whereas both effects did not appear after intra-arterial administration of even a fourfold dose of bradykinin. Opening of the chest wall increased at least fourfold the intravenous threshold dose of bradykinin without affecting the response to the potent bronchoconstrictor agent histamine Vagotomy did not significantly lessen the response of the lungs to bradykinin

Regarding the inhibitory influence of calcium acetylsalicylate on the increased resistance to inflation of guinea-pig lungs *in vivo* produced by bradykinin in our hands the potency of this analgesic

was much lower than that found by COLLIER and SHORLEY (1960). In guinea pigs anaesthetized with urethane we found after an intravenous dose of mg of calcium acetylsalicylate per kg a 4 to 8-fold increase of the threshold dose of bradykinin, whereas a second dose of 8 mg of this drug only increased the threshold of bradykinin by a factor In our experiments both opening of the chest wall and vagotomy considerably lessened the antibradykinin potency of calcium acetylsalicylate.

From these experiments it is concluded that the increase in resistance to inflation of lungs in vivo after small intravenous doses of bradykinin is not due to bronchoconstriction but probably caused by a change in lung compliance. The inhibitory influence of calcium acetylsalicylate on this effect of bradykinin probably arises through an indirect effect on the lung vessels.

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E J ARIËNS *The receptor theory and the relation between structure and action*

Department of Pharmacology University of Nijmegen

The biological effects of drugs are the resultant of the physico-chemical interaction between drug molecules and certain molecules in the biological object. This implies that the physico-chemical properties of a drug and therefore its structure are determinant for the biological activity or in other words that there will be a relation between structure and action. If such a relationship is not observed this may be the consequence of the fact that the structural formulae used represent only very poorly the physico-chemical properties of the drug Another reason may be the ease with which similar pharmacological phenomena are assumed to be comparable A relation

between structure and action can only be expected if identical actions are involved which implies actions induced on identical receptors One would therefore expect a structure-activity relationship between agonists and their competitive antagonists The facts, however are contradictory there is hardly any relation between the structure of histamine and the antihistamines arterenol and the alpha-sympatholytics and acetylcholine and many of the anticholinergics. The study of homologous series of derivatives of histamine arterenol and acetylcholine in which a gradual change from an agonistic to a competitive antagonistic action takes place indicates that the competitive antagonists although blocking the receptors for the agonists do not or only partially occupy the receptors of the agonists They are mainly bound on assessory receptor areas located in the close vicinity of the receptor area for the agonist

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ELLENBROEK, B W J J Pharm. Pharmacol. 17; 393-404 (1965)

H H Beneken Kolmer and F Kreuzer *Continuous polarographic recording of oxygen pressure in respiratory air of man*

Dept of Physiology University of Nijmegen

Whereas the polarographic recording of oxygen pressure in respiratory air has long been performed in our animal experimentation an analogous procedure is described here for man An improved modification of our catheter P_{O_2} electrode (KREUZER *et al* J Appl Physiol 15 1157(1960)) has been used with a response time of 0 2 sec for 95% deflection The respiratory air was continuously suctioned from the mouthpiece of the subject and led first through the pickup of a conventional infrared CO_2 meter and a few cm beyond it through a plastic tube holding the catheter P_{O_2} electrode Since the temperature difference between inspired and expired air had to be equilibrated in view of the temperature sensitivity of the polarographic electrodes the CO_2 unit was kept at 32°C in order to achieve a constant temperature of the terminal gas Comparison between the polarographic readings and simultaneous analyses with the Scholander apparatus showed a satisfactory agreement Further

more it was found that the form of the P_{O_2} curve (as well as that of the CO_2 curve) depended on the rate of gas flow along the electrode. Low flow rates yielded too steep a rise whereas high flow rates resulted in a horizontal plateau. With appropriate adjustment of the flow rate it was possible to obtain an adequate plateau which provided a correct endtidal polarographic reading in comparison with Scholander analysis.

Adrienne J M van Beusekom Kits van Heijningen

1,2 Diphosphoenolpyruvate a new intermediate in the carbohydrate metabolism of rat diaphragm

Department of Physiology University of Amsterdam

Rat diaphragms were incubated with [14]C-glucose and the media chromatographed on paper An unknown intermediate of glucose metabolism was found. The barium salt of this compound was isolated in sufficient quantities through column chromatography as described by KHYM and COHN (1953) A carbonyl group a very labile and a stable phosphorus group could be demonstrated in a ratio of 1 1 1

Mild hydrolysis at 80° and pH 3 5 removes the labile phosphorus group Infrared spectra of the barium salts of this partly hydrolysed product and the original compound have been compared with spectra of 3-phosphoglyceraldehyde, 3-phosphoglycerate 2 phosphoglycerate, 3-phosphohydroxypyruvate hydroxypyruvate 2 phosphoenolpyruvate and acetylphosphate There is a good agreement between the spectra of 2 phosphoenolpyruvate and the product of partial hydrolysis. In comparison with these spectra, the spectrum of the original compound shows the same alterations in the carboxyl group bands as does acetylphosphate, suggesting that the second phosphate group is bound to the carboxyl group

It could be demonstrated enzymatically that hydrolytic removal of the labile phosphorus produces 2 phosphoenolpyruvate whereas upon more drastic hydrolysis pyruvate is formed.

It is concluded that the unknown compound is 1,2-diphosphoenol pyruvate.

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B Bink J Timmers and P van Leeuwen *The aerobic capacity in men aged 20 to 60*

Netherlands Institute for Preventive Medicine Leiden

In 258 men aged 20 to 60 years working with a municipal cleansing department the maximal oxygen intake was determined Ninety-six of them were dustbin loaders and had a mean caloric expenditure of 2140 kcal each daily working time of about eight hours

The capacity of the oxygen transport system – ventilation diffusion-circulation – can be measured from the maximum oxygen intake or aerobic capacity BINKHORST and VAN LEEUWEN (1963) compared the classical method of direct measurement of the aerobic capacity (HILL, 1927 ROBINSON 1939 ÅSTRAND 1952) in which at least three but in most cases many more measuring-sessions are needed with a single-session method based on several repeated measurements of the oxygen intake during a test with continuously increasing load The result of this investigation justified the use of the single-session method throughout our present study

The opportunity to observe the pattern of the electrocardiogram during the testing procedure with increasing load is an additional advantage as the exercise test may be stopped as soon as pathological changes occur

Prior to the exercise test a medical and an occupational history were taken and a physical examination including spirometry and electrocardiography at rest was performed As a result of these preliminary observations we were obliged to give up the exercise test in a few cases

Each exercise test started with two minutes of cycling without a load Thereafter the load increased each minute by 10 watts. Recorded were heart rate blood pressure electrocardiogram ventilation minute volume respiratory rate tidal volume oxygen intake and carbon dioxide output

An example of measurement is shown in Table 1 and Fig 1 Heart rate and oxygen intake tend to level off towards the end of the exercise test

At regular intervals the expiratory minute volume was measured by collection of expired air in Douglas bags At higher loads the expired air was analysed according to the Haldane method In the period prior to final effort Douglas bags were filled during each

TABLE 1

Data obtained during exercise on a bicycle ergometer in a 27 year-old man.

LOAD watts	Pf	S mm Hg	ECG	$\dot{V}$ l/min (BTPS)	f	V l (BTPS)	$\dot{V}_{O_2}$ l/min (STPD)	$\dot{V}_{CO_2}$ l/min (STPD)
rest	94	124/92		10.43	12	0.89	0.33	
rest	100							
0	104	130/90						
0	100							
12.0	97	144/98						
22.0	102							
31.5	103	140/100						
40.5	110							
49.5	106	160/90		10.84	15	0.72		
58.5	122		N					
67.5	130	148/100	O					
76.5	140		R					
85.0	139	162/100	M					
93.5	133		A	33.11	18	1.81	1.53	0.96
103.0	149	152/100	L					
112.0	167							
122.0	172	172/94						
133.5	176							
144.0	182			68.63	25	2.71	2.03	1.53
154.5	189							
165.0	194	190/90						
174.5	198			92.07	33	2.48	2.44	2.04
183.0	196			86.1			2.45	2.22

at consecutive minute. Filling of the last Douglas bag was stopped on the moment the subject gave up. If the filling of the last Douglas bag had not exceeded 0 4 minute the last but one was considered as the last Douglas bag At a filling time longer than 0 4 minute the value was converted into 1 minute. The highest oxygen intake per minute thus determined was considered as the aerobic capacity

The maximal oxygen intake of these 258 men between 20 and 68 years old plotted against age is shown in Fig 2. The results have been grouped in five-year periods in order to calculate the 90% confidence intervals of the observations and of the mean. The regression equation from these data was

$\dot{V}_{O_2\,max} = 3\ 90 - 0.0325$ age in years.

B Bink J Timmers and P van Leeuwen *The aerobic capacity in men aged 20 to 66*

Netherlands Institute for Preventive Medicine Leiden

In 258 men aged 20 to 66 years working with a municipal cleansing department the maximal oxygen intake was determined Ninety-six of them were dustbin loaders and had a mean caloric expenditure of 2140 kcal each daily working time of about eight hours.

The capacity of the oxygen transport system – ventilation diffusion-circulation – can be measured from the maximum oxygen intake or aerobic capacity BINKHORST and VAN LEEUWEN (1963) compared the classical method of direct measurement of the aerobic capacity (HILL, 1927 ROBINSON 1938 ÅSTRAND 1952) in which at least three but in most cases many more measuring sessions are needed with a single-session method based on several repeated measurements of the oxygen intake during a test with continuously increasing load The result of this investigation justified the use of the single-session method throughout our present study

The opportunity to observe the pattern of the electrocardiogram during the testing procedure with increasing load is an additional advantage as the exercise test may be stopped as soon as pathological changes occur

Prior to the exercise test a medical and an occupational history were taken and a physical examination including spirometry and electrocardiography at rest was performed As a result of these preliminary observations we were obliged to give up the exercise test in a few cases.

Each exercise test started with two minutes of cycling without a load. Thereafter the load increased each minute by 10 watts Recorded were heart rate blood pressure electrocardiogram ventilation minute volume respiratory rate tidal volume oxygen intake and carbon dioxide output

An example of measurement is shown in Table 1 and Fig 1 Heart rate and oxygen intake tend to level off towards the end of the exercise test

At regular intervals the expiratory minute volume was measured by collection of expired air in Douglas bags. At higher loads the expired air was analysed according to the Haldane method In the period prior to final effort Douglas bags were filled during each

In 170 of these 258 measurements the investigators were convinced that the subject persisted until his real maximum effort. The relation between aerobic capacity and age length and body weight in these cases was especially examined. Based on these results a multiple regression equation was calculated

$\dot{V}_{O_2\,max} = -0.051 - 0.0279$ age (in years) $+ 0.0184$ height (in cm) $+$ 0.0081 weight (in kg).

Multiple correlation coefficient $R = 0.71$ *s.e.e.* $= 0.398$.

Analysis of variance for partial regression coefficients

Variables	*SS*	*DF*	*MS*	*F*	*P*
Age	16.6435	1		105.23	<<0.001
Height	1.9263	1		12.18	<0.01
Weight	0.8283	1		5.24	<0.03
Residue	26.2547	166	0.15816		

From this study it may be concluded that the aerobic capacity of adult men is largely influenced by their age but also by their height and body weight.

SUMMARY

In 258 men between 20 and 66 years old with considerable professional physical activities, the maximal oxygen intake was determined during a continuously increasing load on a bicycle ergometer The load increased each minute by 10 watts. In 170 subjects there was evidence of maximum effort.

The relation between aerobic capacity and age length and body weight within this group is given in a regression equation.

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Fig 1

Fig 2

L N Bouman and A E Becker *Structure and localization of the sino-auricular node of the rabbit*

Dept of Physiology and Dept of Path. Anatomy University of Amsterdam

In previous investigations (BOUMAN *et al* 1963) on the action of the vagal nerves on the pacemaker activity of the s.-a. node of the rabbit, the observation of PAES DE CARVALHO *et al.* (1959) that spontaneously firing fibres (true pacemaker fibres) are found only in a small area within the nodal region was confirmed. In our experiments these fibres were localized at the venous side of the terminal sulcus, centrally between the orifices of the right superior and inferior vena cava. They were grouped together inside a small zone having a surface area of 1–3 mm² From this zone extensions were running in both cranial and caudal direction over a total distance of about 10 mm, containing latent pacemaker fibres.

In order to determine whether any relationship existed between the electrophysiological localization of the pacemaker fibres and the anatomical site of the sino-auricular node, the distribution of true and latent pacemakers in three isolated rabbit atria was mapped out by a large number of micro-electrode impalements. After the experiment the preparations were cut into serial sections, which were stained according to the following techniques

Haematoxylin-eosin van Gieson stain, elastic tissue stain, phosphotungstic acid-haematoxylin Heidenhain s anilin blue stain, periodic acid Schiff with and without diastase preincubation. To exclude artifacts due to the electrophysiological experiment the nodal area was also examined in serial sections of four fresh preparations.

The sino-auricular node was identified between the orifices of the large veins, showing several characteristic structural differences with the atrial musculature

1 The nodal area is rich in nuclei Their number comes to about twice the number seen in cross-sections of atrial muscle.

2 The nodal fibres are fusiform and slender with a diameter of approximatively one-third of the atrial fibres they run individually in a curved fashion, crossing each other in all directions.

3 Inside the nodal fibres the number of myofibrils is relatively small a distinct cross-striation was found only in fibres at the

I L Bonta O J de Vos and W Hondius Boldingh

Biological activity of fluid obtained from rat paw oedema induced by serotonin

Dept of Pharmacological and Biochemical Research N V Organon Oss

Fluid expressed from serotonin induced rat paw oedema caused contractions of the isolated guinea pig ileum and rat uterus. The effect on the guinea pig ileum was blocked by the antihistamine drug phenbenzamine whereas that on the rat uterus was unaffected by atropine partially inhibited by LSD 25 and blocked by phenyl butazone The contractions of the rat uterus induced by the oedema fluid showed a delayed onset and slow rate of development as did contractions induced by synthetic bradykinin The biological activity of the oedema fluid displayed a tendency to decline even when preserved at 0 C Strong acid treatment of the fluid followed by incubation at 37 C and subsequent neutralization resulted in stabilization and additionally a marked enhancement of that part of the biological activity that was resistant to the above-mentioned inhibitors Unlike the guinea pig ileum and rat uterus, the rat duodenum was relaxed by the oedema fluid Addition of chymotrypsin abolished the biological activity These observations may indicate that alongside histamine and serotonin fairly measurable amounts of kininogen and kinin were present in the oedema fluid There remained the question as to whether the increased activity due to acid treatment was the result of an activation of kallikreinogen (the proenzyme of kinin forming kallikrein) or inactivation of the kinin-destroying enzyme kininase To this end the esterase activity of the oedema fluid was tested before and after acid treatment Pilot experiments using Tosyl Arginine Methyl Ester (TAME) as substrate showed no increase in the esterase activity of the acidified oedema fluid This suggests that acid treatment inactivated the kinin-destroying enzyme rather than activating the kinin forming system. In pilot trials, similar results were obtained with fluids collected from other kinds of rat paw oedemas and from the granuloma pouch.

Further experiments, still under way are aimed at investigating the biological activity of inflammatory tissue fluid collected from animals treated with anti inflammatory drugs.

different intensities in the left ear We found that the patterns for different intensities are the same except that the frequencies for which the maxima and minima occur show a slight shift when the intensity in the left ear is changed. Owing to this it can happen that the pitch intensity relation is very different for various frequencies, even for tones that have slightly different frequencies. It is possible that the sign of the pitch change by intensity change is opposite even when the frequencies are only slightly different.

G van den Brink and G A Reijntjes *Visual facilitation I (theory)*

Dept of Neurophysiology University of Groningen

According to current theories of the visual threshold mechanism, a light-flash would be supraliminal when either two or more (depending upon the theory) light quanta are absorbed effectively within a distance and a time within which addition may occur If a supraliminal light-flash of short duration and with a diameter that is large compared with Ricco s area is presented it should be expected that only a small-sized light-effect is observed in a direction corresponding with the location in the retinal image where the required quantum coincidence occurs independent of the amount and the distribution of elicited effects in the retinal image, outside of the addition area where the threshold is exceeded. The experience is, however that a supraliminal large diameter flash at threshold level is usually seen in its entirety This means that the activity elicited in the retinal image that does not join in the process of threshold exceeding, takes part in the perception as soon as somewhere in the retinal image the threshold is exceeded

If this theory of threshold vision is correct, this observation can only be explained by supposing that retinal activity which would be subliminal by itself is facilitated as soon as somewhere in the retinal image a supraliminal effect is present It must then also be possible to make a subliminal large diameter flash visible in its entirety by adding a supraliminal effect. This can only be realized with a statistical procedure which is demonstrated in the following example

A large flash (6°) is presented simultaneously with a small flash (3′) which is located in the centre of the large flash. If the

periphery of the node The fibres are rich in glycogen as compared with ordinary atrial muscle
4 The nodal fibres are embedded in a relatively large amount of connective tissue containing a rich vascular network
Fibres having these characteristics were found in a large area between the venous orifices. They form a mass of tissue having a size of about 4 5 × 2 5 mm² from which extensions are running in both cranial and caudal direction

A comparison of the electrophysiological and the histological findings showed that the true pacemaker fibres were localized at the site of the greatest mass of the sino-auricular node

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G van den Brink *Relation between intensity and pitch*

Dept of Neurophysiology University of Groningen

The commonly accepted conception that pitch increases with increasing intensity for high frequencies and that the reverse occurs for low frequencies is incorrect in its generality The phenomenon that pitch depends upon intensity known as the phenomenon of Broca, is closely related with a rather irregular relation between pitch and frequency

Usually this relation is not equal for both ears, so that tone pulses with equal frequency are sometimes not perceived with equal pitch in both ears (diplacusis)

Diplacusis is measured as a function of the frequency by alternately presenting tone pulses in the two ears with two oscillators For a diplacusis determination the frequency in the left ear is kept constant the frequency in the right ear is adjusted for equal pitch The frequency difference for equal pitch can be measured

We then find a relation between diplacusis and frequency that shows an irregular pattern with a large number of maxima and minima. In the present series of experiments the intensity in the right ear was kept constant. Diplacusis patterns were measured for

PTE was not blocked by pretreatment with atropine methyl nitrate nethalide, or cyproheptadine.

Incubation of PTE with trypsin and chymotrypsin destroyed its hypotensive potency

ACKNOWLEDGEMENTS

The parathyroid extracts were kindly supplied by Eli Lilly Research Laboratories, Indianapolis (Ind.) U.S.A. (Dr H. Henry) and by N V Organon, Oss, the Netherlands (Dr J D H. Homan)

O M J Driessen *The effect of testosterone propionate on the distribution of I^{131} in the thyroidectomized rat*

Department of Pharmacology University of Leiden

Gonadectomized adult female rats were treated with daily subcutaneous injections of 2 mg testosterone propionate (TP) or with solvent oil for 7 days. Then thyroidectomy was performed and one day later about 5 µC of I^{131} was injected intravenously The blood levels of I^{131} were studied for the next ten hours and appeared to be lower in TP treated animals than in controls. Clearance of I^{131} by the kidney was not affected by pretreatment with TP which was in agreement with the finding that also in nephrectomized rats pretreatment with TP caused the blood levels of I^{131} to be lower than in the controls. In further experiments it was found that TP increased the distribution volume of I^{131} as a result of an augmented uptake by the skin of the experimental animals.

B W J Ellenbroek *Drugs and receptors*

Department of Organic Chemistry University of Nijmegen

As it seems likely that the receptors for cholinomimetics and cholinolytics are not entirely identical, it is important to investigate whether the same structural requirements hold for both parasympathomimetics and parasympatholytics.

The use of optically isomeric compounds in studying this offers considerable advantages. The physico-chemical properties of such compounds differ only in very particular ways, so that differences found in pharmacological behaviour can be traced back to differences in the spatial arrangement of their pharmacophoric groups.

probability of seeing each of the flashes is 0 3 if presented separately, the probability of seeing the large flash in its entirety increases to about 0 5 when both flashes are presented while the probability of seeing the small flash is much smaller than can be expected if we do not suppose a facilitation mechanism

The probability of seeing the large flash increases at the expense of seeing the small flash

G A Charbon *A diuretic and hypotensive action of a parathyroid extract*

Department of Pharmacology Faculty of Medicine, University of Utrecht

The rise of the serum calcium level in dogs 17 hours after the injection is used as the official U.S P biological assay for parathyroid extracts (PTE) Little is known about immediate biological effects of PTE Only a phosphaturia has been reported to occur shortly after the administration of the hormone In our hands PTE (2 5 U.S P units/kg) administered intravenously causes a natriuresis in dogs during the first half hour after injection even in the absence of a phosphaturia (Arch Int. Pharmacodyn. Thér 141 1 (1963)) Increased doses also augmented the excretion of H_2O K^+ Ca^{++} inorganic phosphate and creatinine Accordingly this general activation of renal excretory functions by PTE might be due to a general circulatory effect rather than to a specific renal one

An extract of beef parathyroids was used prepared by the method of Rasmussen and Craig (J biol chem 236 759 (1961)) Intravenously given PTE appeared to have a hypotensive effect in cats and rabbits within one minute after injection The size of the decrease was dose-dependent (3–81 units/kg) The effect of the largest dose lasted more than one hour

Ligation of the renal hili of the coeliac artery and the superior mesenteric vein or of the abdominal aorta and inferior caval vein near the diaphragm did not prevent the hypotensive action of PTE in cats Since the heart rate was increased and the contraction force of the right ventricle was augmented or unchanged at least never diminished the hypotensive action of PTE is probably due to a diminished peripheral vascular resistance The hypotensive action of

line and noradrenaline is of a biphasic type The compound also has local anaesthetic properties.

In many respects the activity of hepzidine resembles on the one hand that of antidepressants like imipramine and amitriptyline and that of antiparkinson agents like orphenadrine on the other

E D Gerlings G Jambroes L N Bouman and P A. Biersteker *The action of the autonomic nerves on the contraction force of the ventricles in the isolated rabbit heart*

Department of Physiology University of Amsterdam

In a previous publication (GERLINGS and BOUMAN 1965) we have shown that the decrease in ventricular contraction force during vagal stimulation in the isolated, perfused rabbit heart is the direct result of the decrease of heart frequency If the frequency is kept constant by artificial pacing the contraction force of the right and left ventricle is not altered during vagal stimulation.

Under the same experimental conditions we have studied the influence of sympathetic nerve stimulation on the ventricular contraction force. The contraction force was measured isovolumetrically by means of water filled rubber balloons which were placed in the ventricles via small incisions in the apex. The pressure in the balloons was measured by "Elema" pressure transducers and recorded on a cardiograph.

During stimulation of one of the cardiac branches of the right stellate ganglion, inconsistent results were obtained These could be explained by the presence of parasympathetic fibres, as stimulation of the stellate ganglion branches led only to an increase in both heart rate and contraction force when atropine-sulphate had been added in a concentration of 3 micrograms per ml perfusion fluid.

To exclude the influence of heart frequency the heart was artificially paced at a frequency just above the maximum obtained during sympathetic nerve stimulation. Even when the heart rate was kept constant in this way stimulation of the sympathetic nerve resulted in an increase of ventricular contraction force.

The results of these experiments indicate that the influence of vagal stimulation is limited to a change in heart rate, while sympathetic stimulation increases both the rate and contraction force It

The optical antipodes of the mimetic drug acetyl β-methylcholine (Mecholin) differ greatly in potency If in this compound the acetyl group is replaced by acyl residues of increasing molecular weight the agonistic character of the esters gradually disappears accompanied by a sharp decrease in affinity However by introducing still heavier acyl groups especially those with ring structures and polar OH groups esters are obtained which behave as competitive antagonists of the starting compound and which have high affinities It is very interesting to note that the optical antipodes of diphenylacetic and benzilic acid esters which differ in the configuration of the β-methylcholine moiety vary only slightly in potency so their stereospecificity is low If however an asymmetrical carbon atom is also introduced in the acyl group as is the case with α methyltropic and hexahydrobenzilic acid then there is again a great difference in potency between the esters having opposite configuration in the acid moiety The affinity is independent of the configuration of the choline moiety in clear contrast with Mecholine

It can be concluded that the receptors for cholinomimetics and cholinolytics are situated close together but have characteristics by which they can be clearly differentiated from each other

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A B H Funcke H M Tersteege W J Louwerse D Mulder and W Th Nauta *Pharmacology of* 4-[(10 11-*dihydro-5H-dibenzo[a d]cyclohepten-5-yl*) *oxy*] 1 *methylpiperidine hydrogen maleate hep idine hydrogen maleate* (*BS* 7051)

Research Department N V Koninklijke Pharmaceutische Fabrieken v/h Brocades-Stheeman en Pharmacia Looiersgracht 27 30 *Amsterdam*

Hepzidine hydrogen maleate possesses central and peripheral antiacetylcholine and antihistamine activity besides antiserotonin and spasmolytic properties It further has stimulating activity as appears from increased locomotor activity in mice stimulation of cats, antagonism to reserpine tetrabenazine chlorpromazine bulbocapnine and thiopropazate It does not antagonize barbiturate-induced respiratory arrest alcohol narcosis is potentiated

The interaction of hepzidine with the pressor response of adrena

From these data oxygen intake $\dot{V}_{O_2}$ and carbon dioxide output $\dot{V}_{CO_2}$ were calculated. Oxygen and carbon dioxide contents of the mixed venous ($C_{\bar{V}O_2}$ and $C_{\bar{V}CO_2}$) and arterial (C_{AO_2} and C_{ACO_2}) blood samples were measured with a Thomas-Van Slyke apparatus using the method described by BARTELS *et al* (1959) The standard deviation calculated from 36 duplicate measurements was 0 15 vol% for O_2 corresponding to a coefficient of variation of 0 98 % and 0.285 vol% for CO_2, corresponding to a coefficient of variation of 0 61%. All values were converted to standard conditions (STPD) and the cardiac output was calculated by means of the equations

$$Q(O_2) = \frac{\dot{V}_{O_2}}{C_{AO_2} - C_{\bar{V}O_2}} \quad (1)$$

and

$$Q(CO_2) = \frac{\dot{V}_{CO_2}}{C_{\bar{V}CO_2} - C_{ACO_2}} \quad (2)$$

The results are shown in Table 1

Causes of the large differences found between the two methods may be summarized as follows.

1 As the amount of CO_2 in blood is high the error in the determination of the CO_2 content of the arterial and mixed venous samples bears strongly on the veno-arterial CO_2 difference It may be shown that an error of 1% in the CO_2 content of both samples causes an error of 25 % or more in the veno-arterial CO_2 difference As the amount of oxygen in blood is slight compared to the CO_2 content errors in the determination of the oxygen content will have considerably less influence on the arterio-venous oxygen difference. This error is generally less than 10 %.

2 The veno-arterial CO_2 difference is smaller than the arteriovenous O_2 difference and thus is more strongly influenced by errors in the blood gas measurements

3 Several other causes, among which are sampling errors (VISSCHER and JOHNSON 1953), errors due to wrong presuppositions in the Fick principle (STOW 1954 WOOD *et al.*, 1954) and errors due to the absence of a steady state during the procedure

Owing to the factors 1 and 2 a difference between $Q(O_2)$ and

can thus be concluded that by simultaneous action of vagal and sympathetic nerves the frequency and the contraction force of the heart can be independently regulated to a certain degree

This conclusion is further supported by the occurrence of an increase in the contraction force during a constant spontaneous heart rate in those preparations where the cardiac branch of the stellate ganglion contained vagal fibres.

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F ten Hoor *Simultaneous measurement of cardiac output in man using the direct Fick procedure with oxygen and carbon dioxide as indicators*

Laboratory of chemical physiology University of Groningen

Since Fick published his very simple principle for the measurement of blood flow numerous cardiac output determinations have been performed using the direct O_2-Fick However the direct CO_2-Fick has only occasionally been used (ZUNTZ and HAGEMANN 1898 COURNAND 1945 COURNAND *et al* 1945) Some authors indicate that the CO_2-Fick is not as accurate as the O_2-Fick owing to the fact that small changes in ventilation may cause considerable changes in CO_2 exhalation (COURNAND 1945 HAMILTON 1962 GUYTON 1963) However experimental data concerning the direct CO_2-Fick are scanty

Simultaneous measurements of cardiac output with both the O_2 and CO_2-Fick have been performed during cardiac catheterization in 36 patients the following procedures being used After the introduction of a cardiac catheter via a cubital vein into the pulmonary artery and of an arterial needle into a brachial or femoral artery the patient was connected to a mouth piece with respiration valves. Expired air was collected in a Douglas bag during an accurately measured interval of 5 to 6 min During the same time-interval mixed venous and arterial blood samples were obtained in heparinized syringes rubber stoppers prevented air contact A sample of the expired air was analyzed with a Haldane apparatus and the total amount of expired air was measured in a dry gasmeter[1])

[1]) Manufactured by Motorfabriek Dordrecht Dordrecht The Netherlands

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J. E. Hueting and R. J. L. Tuyl Schuitemaker

Some reactions during the transition from rest to physical work

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One can roughly distinguish three views concerning the adaptation of the circulation and ventilation systems to physical work.

The responses (Rs) of these systems some time after the onset of muscular work can be mainly considered as a consequence of local changes in the periphery, as Starling and Haldane did.

However, Krogh and Linhard, Cannon and after them many others, such as Jouvet in France and Rushmer in the United States already stressed the fact that cardio-respiratory Rs appear too quickly after the start as to be caused by bloodborne agencies and local mechanical factors. According to these authors, nervous actions must be responsible for the promptness of these Rs.

It was especially the disciples of the Russian Bykov-school who on closer examination, demonstrated changes in vegetative systems on signals occurring in advance of the actual effort, so-called conditional reflexes. A systematic study with quantitative representation and evaluation of these anticipatory cardio-respiratory Rs, however, remained to be undertaken. This was the aim of our study.

In eight non-trained subjects (Os) investigated on 12 days in succession during an ergometric experiment, we studied some Rs on stimuli signalling exercise during the last minute of rest (lmr) in

$Q(CO_2)$ up to about 30% may be expected A larger difference is probably due to the absence of a steady state during the measurements.

TABLE I

Comparison between simultaneous measurements of $Q(O_2)$ and $Q(CO_2)$

No	$Q(O_2)$ (l/min)	$Q(CO_2)$ (l/min)	$\frac{Q(CO_2) - Q(O_2)}{Q(O_2)} \times 100\%$
63008	6 3	7 8	+23.8
6301	7 0	0 1	−19 8
63013	5 0	5 0	0
63018	~ 1	7 3	+ *.8
630°0	4 9	4.8	− 0
630*3	8.5	6 1	− 9
630 6	7.3	9 7	+3° 8
63030	13 4	1 1	− 9 7
63035	6.3	0 6	+ 4 8
63038	5 4	4.3	−.2 *
63048	13 0	9 7	−25 4
63050	14.3	1* 1	−15 4
63054	4 4	4 6	+ 4 5
63061	8.9	14 0	+07.5
63064	6..	8..	+3 .3
6307..	6.7	7.3	+ 9 0
63074	10 0	8 8	−1 0
63080	11 9	10 0	− 8 4
64005	4	7..	+ 1 5
64008	1	1.2	0
64015	6.8	6 0	− 11 8
64010	11..	1 6	+ 11 5
640°*	13.0	13 0	0
64030	5 9	4	+ 78 0
64044	12 7	22 8	+ 79 0
64048	5 8	0	+ 15 6
6405	7 6	11 4	+ 50 0
64054	0 6	10	+ 54.5
64081	4 4	13 0	+195 0
64130	4 3	7 4	+ 7 0
64183	3 6	4 0	+ 27 7
64186	1.8	1 9	+ 5 6
64193	6.6	6.5	− 1.5
64218	3 8	6 4	+ 68 4
64222	5 1	5 1	0
64235	5.8	5 0	− 14 6

cisternal pressure the EEG the ECG EMG and respiration were permanently recorded.

As soon as the I P was raised above the arterial pressure, slowing of the EEG was noticed. With the increase of the I.P the blood pressure rose at the same time After an abrupt excessive elevation of the I.P the following could be noticed 1 A sharp rise of the blood pressure after an initial latent phase of 5–10 sec 2. an increase in the venous pressure 3. a dangerous irregularity of the heart rhythm, 40 sec after the elevation of the I.P lasting several minutes 4. typical EEG changes. Following a latent phase of 25 sec, a 5 sec period of fast activity followed by a phase of depression (10 sec) was registered. The EEG became totally flat, 40 sec after the elevation of the I.P When the I.P sank below the arterial pressure a restoration of the EEG was seen, after a phase of periodic burst activity The EEG either became entirely normal or it remained slower than at the beginning This was dependent on the duration of the intracranial hypertension. 5 The respiration became faster at the beginning more slowly thereafter to cease entirely during the EEG flatness.

About 30 sec after the elevation of the I.P opisthotonus developed followed by extension of the legs. 80 sec later fasciculation of the muscles of the whole body was noticed which lasted 3 min. As soon as the EEG was flat, the function of the C.N.S. ceased, the animals becoming deeply unconscious, immobile atonic with lost reflexes. This condition was reversible after a short period of intracranial hypertension.

When the I.P was maintained at a high level during *0–30 minutes, the animals had to be kept alive artificially in the state of a heart lung preparation, until the heart finally stopped and/or pulmonary oedema developed, in spite of resuscitation (arterenol etc.). It was evident that the heart had suffered from the excessive arterial hypertension, following the vasopressor response after acute elevation of the I.P This was the reason why the animals survived no longer than 6–7 hours.

Suppression of the vasopressor response by the ganglionic blocking action of trimetaphan camphorsulfonate (arfonad) lengthened the survival time considerably (64 hours)

On post mortem examination the brains of the dogs were found to be swollen, with broadened convolutions and flattened sulci The

relation to the minute s rest before and the first minute s exercise (fme) in relation to the lmr

During the lmr Os showed very significant increases in heart frequency (0001 < P < 01 depending on the individuals) These Rs were followed by a further and more stable increase during the fme (all Os P < 0001)

During the lmr Os showed consistent increases in respiration minute volume (0001 < P < 03) During the fme a still more stable pattern of increase was found (all Os P < 0001)

Regarding oxygen consumption increases were found in two Os during the lmr (P < 0001) *The other Os showed a variety of Rs* with too great standard deviations to become significant. The Rs during the fme tended to be more consistent however the pattern remained labile

These seemingly preparatory Rs could not be brought about neither during very light work on the bicycle-ergometer in steady state nor during a five minutes recovery period

One may conclude that during the transitional stages from rest to work adapted activity three mechanisms take part in the integrated R of the organism a central conditional reflex mechanism a central unconditional reflex mechanism and peripheral mechanisms with local chemical and mechanical determinants.

W Kramer and J A Tuynman *Acute increase in intracranial pressure an experimental investigation*

Neurologic Department University Hospital Leyden

In order to get some insight into the mechanism of the intravital death of the brain in patients following cerebral injury 21 experiments on 18 dogs were performed It was necessary to find a method which made it possible 1 to damage the brain exclusively 2 to quantify the lesions 3 to imitate the so-called state of decamimation in man 4 to study the alterations of the brain during different stages of the experiment 5 to get a conception of the pathogenesis of the lesions.

These prerequisites were fulfilled by raising the intracranial pressure (I P) in dogs by *continuously injecting fluid under high* pressure after insertion of a needle into the cisterna magna. The

volume decrease which can be measured with the straingage plethysmograph originally described by WHITNEY (1953) and modified by one of us. The relation between pressure and volume at the place of measurement has to be known and can be determined. The measured volume changes may now be converted into pressure changes. A detailed description of this indirect method was presented.

The direct bloody method and the indirect unbloody method were compared in 20 patients. A good agreement between the two methods was found.

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R S Leeuwin *The influence of androgenic and anabolic compounds on pseudocholinesterase activity in the liver and serum of the rat*

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Immature male and female rats have identical pseudocholinesterase activities in liver and serum. In male rats the enzyme activity declines sharply on maturation, whereas in female rats the activity rises steadily with age (LEEUWIN 1963a 1963b). Orchidectomy of adult males results in an increase of the pseudocholinesterase activity to the immature level and subsequent testosterone administration restores the activity to normal.

Three androgenic-anabolic steroids have been studied as to their effects on pseudo cholinesterase activities in liver and serum of male castrates, viz. testosterone-propionate (TP) testosterone-phenyl propionate (TPP) and nor-testosterone-phenyl propionate (NTPP) Judging from the results of levator ani assays (HERSHBERGER et al 1932 OVERBEEK and DE VISSER, 1957 EDGREN 1963) the latter compound is known to possess a more favourable anabolic-androgenic ratio than the two former compounds. This was confirmed in our experiments and moreover direct determinations in liver and serum showed an elevated protein level in animals treated with the nor-derivative. Liver protein was isolated according to CLELAND and SLATER (1953) and the protein content was determined according to the biuret method (GORNALL et al.,

tissue was oedematous whereas the nerve cells showed regressive changes, clearly visible 5–6 hours after the elevation of the I.P Identical histological alterations were found in normal cerebral tissue of dogs, kept in a saline solution at body temperature during the same period (5–6 hours) The autolysis *in vivo* and *in vitro* was studied with a variety of histochemical techniques (R. G J Willighagen M D) the most important finding being a decrease of 5 nucleotidase activity

Following rapid and excessive increase of the I P the brain will be damaged by mechanical forces further by strangulation and asphyxia and also by stagnation of blood and hypoxia. This leads to the development of cerebral oedema, which in turn tends to increase the I P followed by a further destruction of nerve cells and of the blood brain barrier On the other hand an acute elevation of the I P will elicit a vasopressor response outside the brain with arterial hypertension leading to decompensation of the heart with stagnation of blood in the lungs and in the right side of the heart. These reactions may be followed by pulmonary oedema and/or by cerebral oedema After the death of the brain autolytic processes became active just as in cerebral tissue in a saline solution

It is clear that these experiments do not give us full insight into the pathogenesis of the lesions of every human case but they provide us with some general principles which are valid both in human and animal pathology

J P Kuiper and A J M Brakkee *A new bloodless method for determining the peripheral venous pressure*

Departments of Dermatology and of Medical Physics of the R C University Nijmegen

The venous stasis syndrome cannot be appreciated objectively without a characterization of the venous muscle pumps. The capacity of these pump-mechanisms may be evaluated quantitatively by the determination of the venous pressure The decrease of the peripheral venous pressure, caused by muscular exercise (e.g walking movements) and determined in a distal vein of the foot, is a measure of the function of the venous pumps

This pressure decrease in the venous system is attended by a

llergic reactions to the emulsifier or to its ever-present byproduct I. A. adduct.

In the experiments conducted by Lammers and by us, the pigs id not show papular eruptions during several months of feeding. Furthermore in M.A. 18-fed animals allergy to M.A. 18 could not be proved by intracutaneous injections of M.A. 18. Every pig fed or not fed with M.A. 18, reacted exactly the same The first i.c. injection of 0 2 cc of 1% emulsion of M.A. 18 gave rise after 6–8 days to a red palpable infiltrate at the site of the injection the second and following i.c. injections resulted in a similar infiltrate after 1–2 days. If allergy had been caused by the intestinal route, it could be expected that the first i.c. injection in the M.A. 18-fed pigs had given an infiltrate much earlier than after 6–8 days.

Human beings, patients and controls, reacted in the same way to i.c. injection of M.A. 18 emulsions an infiltrate or a papule after 1– weeks on the site of the injection.

The conclusion from Lammers and our experiments is that allergy to M.A. 18 and/or M.A. adduct can be provoked *e.g* by intracutaneous injections, but not orally This is in contrast with the experiments of Mali c.s. and their explanation of the papular eruptions in M.A. 18-fed pigs. A second batch of pigs was i.c. injected with 1% M.A. 18, freed from the M.A. adduct.[1]) With this no allergic papules could be provoked Probably the M.A. adduct causes the allergic reactions after intracutaneous application. This M.A. adduct contains an anhydride, which can bind itself to proteins. On theoretical grounds Mali c.s. considered the M.A. adduct as the hapten responsible for allergic reactions.

In our opinion the experiments so far have failed to prove that M.A. 18 given orally can provoke allergy to one of its own components. Our working hypothesis is that the emulsifier M.E. 18 may intensify the absorption of some compounds, one of which might cause skin-eruptions in man.

) W thank Prof Boldingh and his co-workers of the Research Laboratory of Unilever (Vlaardingen) for the difficult and time-consuming work of preparing this purified emulsifier

1949) TP and TPP depressed the enzyme level equally effectively when administered in daily doses of 1 mg during ten days. At that dose level the effect of the nor-derivative was much weaker Dose-response relations indicated that a similar response to that of 1 mg of TP could only be obtained when NTPP had been administered in daily doses of 4 mg during ten days At that dose level the androgenic effect on the seminal vesicles is still weaker than the effect of 1 mg TP

It is concluded that the inhibiting effect of the compounds investigated on the pseudocholinesterase activity is correlated to their androgenic potency

(To be published in detail in Acta Endocrinologica)

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L van der Lugt and H Essovold *Some experiments with the emulsifier M E 18*

The emulsifier M E 18 was added to several brands of margarine in Western Germany and The Netherlands respectively in 1958 and 1960 In both countries the introduction of this emulsifier was promptly followed by an explosive epidemic of erythema multiforme-like eruptions.

We will not discuss the controversial medical opinions concerning the direct cause of this disease here but we will confine ourselves to the results of some experiments on pigs

Pigs were experimentally fed with suspected margarine by Mali and co-workers by Lammers (R I V Utrecht) and by us.

Mali c.s described papular eruptions in pigs after several months of feeding the margarine The authors interpreted these eruptions as

the recording of the contractions of the leg muscles was of no use for the study of the recovery of the vital respiratory muscles.

G A Mook P Osypka R E Sturm and F H Wood
Light reflection measurements on blood by fiber-optic catheter

Lab Veg Physiology University of Groningen, The Netherlands and Mayo Graduate School of Medicine Rochester Minnesota, U.S.A

The application of fiber-optics, mounted in a cardiac catheter to record oxygen saturation and dye dilution curves by measuring the light reflected by blood from intracardiac and other intravascular sites, was introduced by POLANYI and HEHIR in 1962 Since large variations in the blood reflection occur especially with changes in blood flow compensation for these non-specific effects must be attained before accurate measurements can be made Therefore a two-color technique must be used.

The instrument used contains a light source (Tungsten lamp 10 V-8 A) illuminating the incident fiber bundle of the catheter This bundle transmits the light to the intravascular tip of the fiber-optic catheter The reflected light is conducted by the output fiber bundle and split by a dichroic mirror The two beams then traverse additional filters before reaching the detecting and compensating photomultiplier tubes (RCA 7102) The catheter used contained 60 fibers per bundle

Fiber-optic reflection spectra of oxygenated and reduced ox blood samples were made with this instrument, the filters being removed using a Beckman DU spectrophotometer (slit width 0.2 mm) as light source These spectra show an isobestic "region" between 840 and 880 nm Transmission spectra of the same blood samples obtained with the same set-up using a second fiber-optic catheter to transilluminate the blood show an isobestic point at about 790 nm. The location of the isobestic point for fiber-optic reflection between 840 and 880 nm was confirmed for dog blood *in vivo* by recording the change of the reflection of arterial blood following nitrogen breathing, using different interference filters.

For oximetry the instrument, therefore incorporates a filter with peak transmission at 880 nm in the compensating channel and a 640 nm filter in the detecting channel. For densitometry of indocyanine green, filters with peak transmission at 800 and 920 nm

E Meeter and O L Wolthuis *The spontaneous recovery of respiration and neuromuscular transmission in the rat after injection of cholinesterase inhibitors*

Medical Biological Laboratory of the National Defence Research Organization TNO Rijswijk (Z H)

Little is known about the length of the period during which artificial respiration (AR) is required to save animals poisoned with various anticholinesterases. Rats (200 g) were anaesthetized with veronal (215 mg/kg) and subsequently injected (s.c) with various doses of DFP sarin or soman Immediately thereafter 10 mg atropine was given (i p) and AR was started At half hour intervals AR was stopped to test for spontaneous breathing The AR time was determined being the time between the injection of the poison and the beginning of that test period in which the animal proved able to breathe unaided for at least 60 minutes.

Each dose of poison was tested in at least 6 animals. With all poisons the AR time increased with the dose. Rats poisoned by 2 and $4 \times LD_{50}$ DFP needed 127 ± 14 and 210 ± 17 minutes of AR respectively With $8 \times LD_{50}$ all animals died from heart failure Sarin poisoned rats, on the other hand could all be saved from $64 \times LD_{50}$ in 150 ± 20 minutes. Resuscitation of soman rats after 2 LD_{50} took 450 ± 61 minutes. After $4 \times LD_{50}$ it took about 10 hours in 3 out of 7 animals, the remaining 4 still needed AR at the end of the experiment, 12 hours after poisoning

In order to correlate the return of spontaneous breathing with the recovery of neuromuscular transmission, in a number of rats the diaphragm was indirectly stimulated and the contractions recorded. A pneumatic method was used which allowed registration of the muscle contractions with chest and abdomen closed during short periods of apnea following forced hyperventilation. At regular intervals tetanic stimulation was performed during 3 sec with 25 50 100 and 200 stimuli per second. It was found that only partial return of the ability to sustain a 50/sec tetanus was needed for adequate spontaneous breathing

Recovery of junctional transmission in the diaphragm was compared with that in the gastrocnemius-soleus muscles. It appeared that the leg muscles could be synchronized with the diaphragm by heating them to the correct temperature. If this was not done

flow This is due to the reflecting properties of the blood which are apparently wavelength dependent. This can easily be demonstrated by measuring fiber-optic reflections at different blood flow rates and plotting the values obtained at the two wavelengths (detecting and compensating photocell outputs) against each other Such a detecting cell/compensating cell line (D/C line) is slightly curved. Obviously for ideal compensation of flow effects, this line must be straight and pass through zero Matters are further complicated by the fact that D/C lines obtained with oxygenated and with reduced blood are curved in opposite directions. Moreover at least for oxygenated blood, the effect of changes in hemoglobin concentration is opposite to the effect of changes in flow rate. Thus, if one succeeds in straightening the D/C line for changes in flow e.g with the aid of an analog computer compensation for changes in hemoglobin concentration has become worse It would seem that for each individual application of the fiber-optic oximeter or densitometer it must be decided which solution to these problems is the most favourable.

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J Offermeier *On the action of 5-Hydroxytryptamine on a sympathetic receptors*

Department of Pharmacology University of Nijmegen

In many cases the effect produced by 5-hydroxytryptamine (5-HT serotonin) when tested on isolated organ systems, is not unequivocal an auto-inhibitive effect is often noticed when employing higher doses. This is the case when 5-HT is tested on the isolated rat uterus, rat fundus strip, etc. (ERSPAMER, 1961).

INNES (1962) reported an action of 5-HT on adrenaline receptors of the cat splenic muscle and FURCHGOTT (1954) mentioned that high doses of 5-HT possibly protect the noradrenaline receptors in the rabbit aorta strip preparation against the action of irreversible receptor blocking-agents, a property shared by substances which interact with the noradrenaline receptors.

are used in the detecting and compensating channels respectively

The fiber optic reflection of blood is very sensitive to changes in hemoglobin concentration In the range of 8 to 20 grams/100 ml the reflection increases almost linearly with the hemoglobin concentration in contrast to findings of almost no change in this range obtained with conventional reflection oximeters (ZIJLSTRA and MOOK 1962) The best compensation for changes in hemoglobin concentration is obtained by taking the ratio of detecting and compensating photocell outputs as proposed by Polanyi This ratio however is not yet completely independent of the hemoglobin concentration

The relation between the ratio of fiber optic reflections at 880 and 640 nm (R^{880}/R^{640}) as used by Polanyi and the oxygen saturation as determined by Van Slyke analysis is non linear although the deviation from linearity is but slight However a good linear relationship is found between the logarithm of R^{640}/R^{880} and the oxygen saturation

The relation between the ratio of fiber-optic reflections at 800 and 920 nm and the indocyanine green concentration was studied in the range from 0 to 40 mg/l Both ratios, R^{920}/R^{800} and R^{800}/R^{920} change non linearly with the dye concentration However from 0 to 20 mg/l the deviation from linearity is but small We prefer to use the R^{800}/R^{920} ratio which is more convenient for analog computation.

Polanyi uses one photomultiplier in his instrument originally to avoid the problem of finding two photocell filter assemblies with the same response characteristics We have checked our instrument for static and dynamic unbalance of the two photocell filter assemblies. Non-specific changes in the intensity of reflected light were produced by varying the distance between the catheter tip and a mirror An analog computer was used for continuous computation of the ratio of the two photocell outputs. The ratio appeared to be independent of considerable changes in the individual photocell outputs, as they follow the fluctuating reflections. Therefore the dynamic and static response characteristics of the photocell filter assemblies are identical at least at the lumination levels at which they are operated in the fiber-optic densitometer or oximeter

However with the catheter tip in blood the ratio changes considerably with changes in light reflection caused by changes in blood

J B Ploem *The formation of ring-shaped mitochondria under the influence of 2,4-dinitrophenol and the vital staining of these structures with tetracycline using fluorescence microscopy with reflected light*

Pharmacological Laboratory University of Amsterdam

The effect of 0 05 mM dinitrophenol on the mitochondria in cells isolated from rat embryos (Re-1 cell line) was studied with the aid of the phase contrast microscope In accordance with the findings of FREDERIC (1954 1958) it was observed that after 1-2 hours of treatment the mitochondria became thicker and longer presented local swellings and often tended to stick together

If the treatment with dinitrophenol lasted longer than 3 hours, we observed that most of the chondriome was present in the form of ring-shaped structures with a diameter of 2-4 μm, showing small local swellings.

Mitochondria can be stained vitally with tetracycline (50 μg/ml 5 min) and show a yellow fluorescence after excitation with violet light, as described by DU BUY and SHOWACRE (1961) Phase contrast microscopy before and after this vital staining, performed in a perfusion chamber showed that the structure of the mitochondria was not affected by this concentration of tetracycline.

When cells were stained longer with higher concentrations of tetracycline (6 h, 100 μg/ml) a swelling of the mitochondria could be observed with phase contrast microscopy (see also JOURNEY and GOLDSTEIN 1963)

For the microphotography of the fluorescence of mitochondria stained with tetracycline very strong violet excitation light is necessary This was isolated from the light of a high pressure mercury arc lamp with a dichroic mirror in combination with reflection microscopy using apochromatic objectives with high N.A. If cells treated with dinitrophenol were stained with tetracycline (50 μg/ml, 5 min), nearly every detail of the deformed mitochondria, visible with the phase contrast microscope could also be observed with fluorescence microscopy

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JOURNEY L. J and M. N GOLDSTEIN, Cancer Res. 23; 551 (1963).

In search for test objects suitable for the evaluation of 5-HT and related substances, the isolated rat vas deferens was used an organ often employed for the evaluation of α-sympathomimetic substances The contractions produced by 5-HT on this organ can be competitively antagonized by α-sympatholytics Different doses of an α-sympatholytic produce the same amount of shift in the dose-response curve of 5 HT as corresponding doses do in the dose-response curve of noradrenaline on the same organ This means that the pA_2 values (ARIËNS and VAN ROSSUM 1957) found for an α-sympatholytic, for instance Droperidol are the same whether the agonist employed be 5-HT or noradrenaline

The rat fundus strip preparation is very sensitive towards 5-HT (VANE 1959) but higher doses of 5-HT produce an auto-inhibitive effect on this test object This auto-inhibitive effect can be competitively antagonized by an α-sympatholytic for instance piperoxane in doses of 10^{-6} or 10^{-5} molar

Serotonin in doses of 10 to 30 μg/kg produces a mixed response on the blood pressure of a cat under pentothal anaesthesia namely a short-lasting pressor response followed by a longer lasting depression of the blood pressure The pressor response can be completely antagonized by an infusion of piperoxane (10–12 mg/kg/h)

The above-mentioned results plead for an action of 5-HT on α-sympathetic receptors whether this is a direct or indirect action remains to be investigated

A marked sensitization of the dose-response curve of 5-HT on the rat fundus strip occurs in the presence of cocaine imipramine or adamantadine Sensitization of the noradrenaline curve on the rat vas deferens is also found with cocaine imipramine (VAN ROSSUM) and adamantadine indicating that perhaps the transport mechanism for noradrenaline and serotonin is similar

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CONCLUSIONS

In work with an oxygen uptake less than 50% of the maximum the heart rate oxygen uptake and lactic acid content of the blood remain at a constant level during an hour s work.

In all experiments in which the subject reached a point of exhaustion, the heart rate was near the maximum. Oxygen uptake and lactic acid were only maximal with the heaviest work-loads, with exhaustion within 8 minutes. Measurements of the aerobic capacity in experiments of longer duration than 8 minutes can be too low

As a tentative conclusion we think that, in spite of a non maximal oxygen uptake in work of longer duration the cardiac output is the limiting factor as the skin circulation increases very considerably in those cases.

H van Rees *The renal excretion of mandelic acid after repeated administration of styrene to rats*

Netherlands Institute for Preventive Medicine, Leiden

An investigation was performed into the renal excretion of mandelic acid as an indication of the elimination of styrene ad ministered to rats.

After a single intraperitoneal injection of 6 mg styrene an ex ponential excretion of the metabolite mandelic acid was found. The biological half time was 3½ hours. Thus in 24 hours more than 90% of the mandelic acid formed out of the styrene is excreted, which amounts to about one third of the dose

After repeated intraperitoneal administration of either 6 mg, 12 mg or 18 mg styrene (once daily in the morning during 4 days) no cumulation occurred, as was concluded from the fact that the daily excretion of mandelic acid did not increase However the fraction of the injected styrene, which is excreted as mandelic acid, was smaller after the highest dose than after both the lower doses. Splitting the excreted mandelic acid into day-portions (from 0 to 8 hours after administration) and night-portions (from 8 to 24 hours after administration) this fraction appeared to be independent of the dose at night, while during daytime it appeared to be smaller after the highest dose than after the other doses.

This discrepancy may be explained by the assumption that during

J Pool[1]) and A Bouhuys [2]) *Exertion of longer duration*[3])

Laboratory of Clinical Physiology Department of Internal Medicine University Hospital Leiden The Netherlands

Exertion of short duration less than one minute is limited by the maximum oxygen debt With exertion of a duration of two to eight minutes the aerobic capacity is the limiting factor

In order to analyse the factors which limit the work of longer duration, a subject was put to work on a bicycle-ergometer at different loads varying from 300 to 1050 kgm/min until exhaustion followed or to a maximum of one hour During and after work, measurements were made of heart rate oxygen uptake ventilation lactic acid and pH which was estimated in arterialized finger blood.

RESULTS

Oxygen uptake and lactic acid content of the blood showed a constant level in work loads up to 1050 kgm/min heart rate also showed a constant level in loads up to 750 kgm/min. At higher work levels, however there was a constant rise of these factors. The subject became exhausted within an hour with work loads of 1050 kgm/min and more

With work loads which could be maintained for 8 minutes or shorter oxygen uptake heart rate and lactic acid reached maximum values. When exhaustion occurred after at least 10 minutes of exertion neither oxygen uptake nor ventilation or lactic acid content reached maximum levels for this subject nor did the pH sink to a very low value Only the heart rate increased to a maximum level in all experiments which led to exhaustion

In an experiment at a working level of 300 kgm/min skin circulation was measured by venous occlusion plethysmography of the finger During the work test the skin circulation increased by a factor 4 in half an hour It is supposed that this increase was necessary for heat regulation

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3) This study was supported by the National Tuberculosis Association — American Trudeau Society

density of elicited effects is greater for equal probability of seeing (Piper s law). This means a higher level of retinal activity which evidently lasts over a longer period.

W J Rietveld *Contribution of various retinal areas to the visual evoked potential in the human cortex*

Department of Physiology University of Leiden

In previous investigations it was found that the occipitocortical response to lightflashes in man is a polyphasic waveform in which several elements can be identified.

A positive A wave at about 30 msec after the flash is followed by several negative B-waves at 50–70 msec, which in their turn are followed by a deep trough (*Γ*) at about 150 msec. The later part of the response consists of a surface-negative wave (*E*) followed by some after-oscillations. In experiments with various luminous discs and rings it was found that the main contribution to the response was furnished by the most central part of the foveal area. The *B*- and *Γ* waves are generated in this area only while the later components originate in the peripheral areas, too.

Furthermore it was found that the *B*-waves signal local illumination level while the later waves, on the other hand, receive integrated information from rather extended areas

Separate illumination of the foveo-parafoveal quadrants showed *B* and *Γ* to be of different origin. The *Γ* wave spreads slowly from its site of origin at a rate suggesting an interneuronal pathway

The behaviour of the components of the visual evoked potential indicates that at least three different systems are involved in its formation.

D Spruit *Variability of the insensible water permeation through the human skin*

Department of Dermatology R.C University of Nijmegen

The diffusion of water through the skin *in vivo* is registered with the aid of an electrolytic water analyser (Gasselt and Vierhout 1963 Crawshaw and Davidson 1959), and found to be constant for about an hour or more This insensible perspiration has even been reported to remain constant for 25 hours (Wagener, 1963). Yet,

the first period after administration of the highest dose the maximal capacity to convert styrene into mandelic acid was approached.

G A Reijntjes and G van den Brink *Visual facilitation II (experiments)*

Dept of Neurophysiology University of Groningen

Temporal and spatial properties of the facilitation mechanism proposed in a previous paper are studied

The experimental arrangement provided two 10 msec lightflashes with variable intensity and variable interval The centres of the flashes were presented 7 temporally from the fovea in the observer s dark adapted right eye

In the first experiment undertaken to study spatial aspects of facilitation a large diameter flash (6°) and a small one (3) were presented simultaneously The probability of seeing the small flash c_s was kept constant that of the large flash c_l was varied between 0 1 and 0 9 The probability of seeing the large flash increases with intensity The number of elicited effects increases then by which the average mutual distance between the effects decreases. The amount of facilitation depends upon distance so that the probability that a subliminal large flash is facilitated by a small flash and becomes visible in its entirety increases with increasing intensity The distance within which facilitation occurs appears to be distinctly greater than the distance within which subliminal effects add up

The second experiment was undertaken to study temporal aspects of facilitation By variation of the interval between the large-diameter flash and the small one it is shown that facilitation is active over a larger interval than addition is.

In the third experiment two bar-shaped stimuli were used. The two bars were perpendicular to each other and formed a cross when perceived together These stimuli were of equal value and yet easily distinguishable. Each of them could facilitate and be facilitated by the other one

Using those stimuli the interval within which facilitation occurs is distinctly greater than in the first experiment This must be due to the fact that the surface of the stimuli is much smaller than the surface of the large circular flash in the first experiments, so that the

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W J B M van de Staak *Use of the heated thermocouple method in measuring the blood-circulation in the skin*

Department of Dermatology R C University of Nijmegen

The surface-applicator as described by Hensel (1956 1959) was modified. Using this applicator good information was obtained about the blood circulation through the skin. *Measurements are based on* the principle of the 'heated thermocouple-method

The transport of heat in the skin is dependent on the conduction through the tissue and on the convection by the circulating blood. *By reducing the arterial circulation to nought (total occlusion)* the contributions of conduction and convection can be separated. In this way it is possible to obtain information about the circulation through the skin.

The measurements revealed significant differences between the circulation of normal individuals, patients with hypertensive ulcers at the legs, and patients with erythrocyanosis crurum puellarum. *The patients* with hypertensive ulcers showed hardly any reaction to occlusion and no reactive hyperaemia after total occlusion. Patients with erythrocyanosis crurum puellarum showed only a minor reaction at occlusion, but the reactive hyperaemia was often fairly normal

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W F H Stroër *On the influence of molecular weight and concentrations of dextrans on the ESR*

Pharmacological Dept of Poviet Products N V., *Amsterdam*

This investigation is the result of the accidental observation that the ESR of blood, mixed 4 1 with dextran of different Mw (40 000 – 70 000 – 140 000 – 200 000) in solutions of 2, 4, 8 and 10 per cent, did not increase regularly with increasing Mw and concentration. This

when measurements are repeated on different days, the results of the values found at exactly the same site of the skin differ very much e g 0 53 0 65 0 81 0 80 0 74 0 74 0 75 0 64 mg water/cm²/h In five individuals the coefficient of variation was found to be about 12% of the average value of the individual insensible perspiration

The water permeation P through the skin—just like through synthetic membranes—is dependent on the absolute temperature (BLANK 1952 MALI 1956) according to

$$\ln P = -E/RT + \ln P_0.$$

The experimental value of ln P plotted against $1/T$ is shown to be very well in accordance with this physical relation in *in vitro* experiments and results into a straight line It is also possible to demonstrate this relation in *in vivo* experiments but not always. Especially when the temperature of the skin is changed slowly deviations from the straight line are registered After the skin has been cooled the temperature gradually increases again The values of the insensible perspiration during this increase are lower than they were during cooling Insensible-perspiration skin-quality has been improved as permeation decreased It is possible to preserve this quality for a short time by quickly lowering the temperature of the skin It is shown from the identical slope of the curve that—though the permeation has been decreased—the activation energy for permeation E is just the same as before A physiological change of the insensible perspiration of the skin is made most probable by the result of this experiment

A gradual decrease of the skin temperature is also accompanied by a change of the permeation quality of the skin Now permeation is becoming higher than might be expected from the physical relation. A similar variation is noted when sweating occurs.

The variation of the permeation quality of the skin may amount to up to 37 % of the average value of the insensible perspiration So changes are quite important and may influence the evaluation of the functional protecting quality of the skin appreciably

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B A Verhey) L C M van Keulen) and P E Voorhoeve)

An extreme form of presynaptic inhibition by Ia Afferents

Dept of Physiology University of Leiden

Presynaptic inhibition in the spinal medulla depends on partial depolarization of afferent fibres diminishing the size of the incoming impulses. These smaller impulses cause a smaller amount of transmitter-substance to be liberated at the synapse.

It is known from the literature that Ia afferents from flexors are the only ones among the muscle-spindle afferents which exert a moderate depolarizing effect upon other muscle afferents.

In previous investigations (VOORHOEVE and VERHEY 1963) it was shown that selective activation of Ia afferents by close arterial injection of succinyl choline (SCh) into flexors caused a depression of monosynaptic reflexes by 25-50%. This depression was due to presynaptic inhibition.

In the present investigation it was found that activation of muscle-spindles in hip flexors and -adductors with SCh caused a total depression of the quadriceps monosynaptic reflex, while monosynaptic reflexes from other muscle afferents were only partially depressed.

Primary afferent depolarization of the quadriceps Ia afferents was apparent by an increase in excitability of these fibres of some 30 % to direct stimulation with a microelectrode in the nucleus (ECCLES *et al.*, 1962).

It is surprising that hip flexor Ia afferents exert this extremely potent presynaptic inhibitory effect only on the quadriceps monosynaptic reflex, especially as they do not seem to have any other specific functional relationship to the quadriceps.

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was ascribed to the formation of complexes between different dextran fractions and the plasma proteins In order to investigate this dex tran BP was fractionated into 9 fractions and solutions of 6 8 and 10 per cent were prepared from each (Mr Gauw)

It was found that erythrocytes coated with dextran molecules, suspended in heparine-plasma, in defibrinated plasma or in serum did not sediment at all or very slowly After treatment with prota mine which sediments selectively fibrinogen a normal ESR was observed It was concluded that fibrinogen plays a role in the coating of the ery s and that dextran-coating blocks the 'receptor" (according to the scheme of Ruhenstroth Bauer) on the membrane of the red corpuscles and so prevents their sedimentation Fibrinogen forms complexes with all the investigated dextran fractions in the three concentrations used None of the other plasma proteins with the exception of a 10% solution and β-globuline combines with all the fractions.

Electrophoretically it was shown that the sediment in plasma, caused by two dextran fractions (Mw 141 000 and 500 000) consisted of fibrinogen β-globuline and praealbumine i e the proteins which —according to Möller and Gramlich—are absorbed on the erythrocyte surface When normal 3 × washed erythrocytes were suspended in serum or in defibrinated plasma mixed with dextran all 3 concen trations sedimented in the same way i e the LMWD sedimented very slowly the HMWD (185 000 and larger) sedimented very quickly and the MMWD (95 000–185 000) formed a transition group This was not due to a lack of fibrinogen but to the thrombocytes used in the clotting process as was concluded from a comparable sequence of ESR s in citrate blood (3 8 per cent tri sodium citrate) and in heparine-blood (only 6 % dextrans) It was shown by several authors that the inhibitory effect of heparin on the coagulation of plasma is in inverse proportion to the number of platelets or amounts of platelet material especially of platelet factor 4 The rather compli cated interactions between blood and heparin are differently influenced when 6 8 or 10 per cent dextran is added This has to be investigated more exactly That dextran internets not only with the plasma proteins but also with blood corpuscles is shown in rats, where it causes mast-cell disruption with release of serotonin and histamin. It was found that these reactions also depended on the Mw of the fractions used and their percentages.

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A A Verveen and H E Derksen *Membrane voltage fluctuations*

Dept of Physiology University of Leiden

The fluctuations of the resting nerve membrane potential of the frog node of Ranvier exhibit a $1/f$ type of frequency spectrum for the range between 1 and 10 000 rad.sec^{-1}

The energy content of this spectrum is related to the flux of potassium ions.

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P Visser and M J W Kuhuwael Tonneman *On a so-called spatial factor preventing interference between learning and relearning codes in an encoding experiment*

Department of Psychophysiology Physiological Laboratory University of Amsterdam

On a form a sequence of 120 characters randomly drawn from the four consonants B – D – N – R, is coded into a number-code by writing under each character a figure according to a given code (e.g. B-D-N-R = 1-2-3-4) Each subject has to encode eight of such forms. After a break 8 new forms are encoded according to a second code e.g. N-D-R-B = 2-4-1-3. In one series of eight forms the time used for each form decreases, which indicates some learning process. The times used for each form of the second series are compared to the corresponding times of the first series. In most of the subjects these times tended to be longer in the second series, which is interpreted as an indication of an interference between the two codes given

The written encoding is replaced by the use of a mechanical

A Versprillo *Acetylcholine and the negative chronotropic effect of adenine derivatives on the frog heart*

Dept of Physiology University of Utrecht

In the appendix of a previous paper (VERSPRILLE, 1965) we concluded that the negative chronotropic effect of adenine derivatives on frog and mammalian hearts probably cannot be explained by a process of deamination of these substances as DRURY and SZENT GYÖRGYI (1929) supposed.

According to experiments of BEZNAK (1951) ATP releases acetylcholine (Ach) from the motor end plates in the M. rectus abdominis of the frog Addition of ATP to the perfusion fluid of this isolated muscle causes an acetylcholine-like contracture which is abolished by d tubocarine and augmented by eserine

Perhaps the negative chronotropic effect of ATP and other adenine derivatives is caused by a release of Ach from the cut efferent vagus-fibres.

We studied the effect of ATP and adenine on frog hearts during perfusion with Ringer s solution and during perfusion with Ringer s solution + atropine (0 1 mg%) The negative chronotropic effect of ATP and adenosine was not influenced by atropine which did completely abolish the depressive effect of 10γ acetylcholine on the frog heart

We concluded that ATP and adenosine do not release acetylcholine from vagus-fibres.

We also repeated the experiments of Beznak In 34 experiments we did not observe any effect of ATP on the M rectus abdominis of the frog not even during perfusion of the muscle with Ringer s solution + eserine

The only difference between Beznak s experiments and ours is the difference in the ATP preparation Beznak used Ba or Ca salt of ATP which was prepared by Szent Györgyi Buchtal or Morgan With Na-oxalate or Na-sulphate he got the Na-salt of ATP We used the dipotassium salt of ATP from N B Co Cleveland Perhaps an impurity in the preparations of Beznak caused the contracture of the M. rectus abdominis. We have no other real explanation for the differences in the results of Beznak and the results we described above

For every muscle length the force exerted can be converted into a percentage of the maximum force at the length at issue. If the force is expressed in this way the same relation is found between the force and the electromyographic activity for all muscle lengths.

O A ter Weeme J W F Beks and E J Ebels *Experimental cerebral oedema*

Department of Neurophysiology Neurosurgical Clinic, Department of Pathology University of Groningen

Different kinds of hypertonic solutions are used in neurosurgical practice in combating cerebral oedema.

In order to gain an insight into the activity of these solutions it is necessary to dispose of laboratory animals with cerebral oedema and to be able to provoke the oedema experimentally One of the methods is local hypothermia of the cerebral cortex.

In our experiments we used adult cats. Cannulas were introduced into an artery and a vein a needle was moved into one of the lateral cerebral ventricles local cooling was applied to the cerebral cortex at the opposite side by means of a thermode, screwed into a trepanation hole

We tried to obtain information about the relation between the intraventricular pressure the arterial bloodpressure and the clinical pathological picture at an increase of the intracranial pressure until the moment of cerebral herniation.

During the gradual increase of the cerebrospinal-fluid pressure respiratory and cardiovascular disturbances occurred.

If no therapeutical measures were taken the cat died of herniation of the brain stem.

The arrangement and the results of some experiments were discussed at the meeting

REFERENCE

BEKS et. al., Increase in intraventricular pressure in cold induced cerebral oedema. Acta Physiol. Pharm. Neer 13; 317 (1965)

device which shows one character at a time of the used randomized sequence of *120* characters B D N R while encoding takes place by pushing one from a line of four push buttons according to the B-D-N R – button (place) code used When this mechanical device is used the interference between the learning or first code and the relearning or second code does not occur From the introspections given by the subjects a hypothesis is induced that the spatial arrangement given by the push buttons prevents the occurrence of interference In order to test this hypothesis in a new group of subjects two series of different forms are tested Half of the number of subjects started with the above-mentioned learning and relearning of the consonant-to figure codes whereas the other half started with forms on which a consonant-to 'place code was given. Under each character of these forms a line of four squares is printed one of which must be checked according to the given code. After an interval of weeks the subjects are tested with the forms they did not have in their first test It is found that the so-called spatial factor thus introduced on the forms prevented the occurrence of interference between learning and relearning codes. With the mechanical device a comparable experiment is done by encoding with one push button only the interference reappears. An analysis of variance gave significance of three factors at a 0 01 level respectively transfer from one session to the second after the break, difference between consonant-to-figure code and consonant-to-place code and interference between learning and relearning

J Vredenbregt W G Koster and J M Westhoff *The relation between the electromyographic activity the force exerted and the length of the m. biceps under static conditions*

Institute of Perception Insulindelaan 2 Eindhoven

The relation between the electromyographic activity and the force exerted by the m biceps, was measured at the wrist under static conditions at different muscle lengths.

The force was varied from zero to maximum effort. It appears that the value of the electromyographic activity increases more than proportionally with respect to the force exerted At a constant myographic activity the force increases *with the length of the* muscle.

For every muscle length the force exerted can be converted into a percentage of the maximum force at the length at issue If the force is expressed in this way the same relation is found between the force and the electromyographic activity for all muscle lengths.

C A ter Weeme J W F Beks and E J Ebels *Experimental cerebral oedema*

Department of Neurophysiology Neurosurgical Clinic, Department of Pathology University of Groningen

Different kinds of hypertonic solutions are used in neurosurgical practice in combating cerebral oedema.

In order to gain an insight into the activity of these solutions it is necessary to dispose of laboratory animals with cerebral oedema and to be able to provoke the oedema experimentally One of the methods is local hypothermia of the cerebral cortex

In our experiments we used adult cats Cannulas were introduced into an artery and a vein a needle was moved into one of the lateral cerebral ventricles local cooling was applied to the cerebral cortex at the opposite side by means of a thermode, screwed into a trepanation hole

We tried to obtain information about the relation between the intraventricular pressure the arterial bloodpressure and the clinical-pathological picture at an increase of the intracranial pressure until the moment of cerebral herniation.

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O L Wolthuis and E M Cohen *The effects of P₂S LüH₆ and TMB₄ on the isolated rat diaphragm treated with tabun or soman*

Medical Biological Laboratory of the National Defence Research Organization TNO Rijswijk Z H

The effects of equimolar concentrations of P₂S LüH₆ and TMB₄ were investigated on the indirectly stimulated isolated diaphragm preparation of the rat inhibited by tabun or soman Additional experiments with the same oximes and inhibitors were performed on intact atropinized rats Oximes were added (injected) either before or after the nerve gases.

The results show differential effects of the three oximes in general P₂S is more active against intoxication with soman whereas TMB₄ and LüH₆ are more effective against poisoning with tabun

The results indicate that neither of the oximes studied is the drug of choice against intoxication with organophosphorus inhibitors in general the final outcome of oxime administration also depending on the structure of the inhibitor involved.

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NETHERLANDS SOCIETY FOR ENDOCRINOLOGY

ABSTRACTS OF PAPERS READ UNDER THE AUSPICES OF THE SOCIETY AT THE SEVENTH FEDERATION MEETING OF MEDICAL-BIOLOGICAL SOCIETIES AMSTERDAM APRIL 14–15 1966

PROGRAMME

H Bal and P G Smelik *Effect of hypothalamic lesions on MSH-content of the intermediate lobe of the pituitary in the rat*

Department of Pharmacology Medical Faculty University of Utrecht The Netherlands

In order to investigate whether hypothalamic structures may be involved in the production of melanocyte-stimulating hormone (MSH), lesions were placed in several parts of the hypothalamus of female rats. After one week the animals were sacrificed, and the posterior lobe (pars intermedia + pars nervosa) was removed and extracted. The MSH content was determined *in vitro* on skin of the lizard Anolis carolinensis, according to the method of BURGERS (1961). As a reference standard synthetic α MSH was used, so that the MSH content could be expressed in μg α MSH-activity/posterior lobe (PL)

The posterior lobe MSH content in 27 sham-operated control animals appeared to be $1\ 09 \pm 0\ 14$ μg/PL.

Lesions destroying the median-eminence-stalk region of the hypothalamus had no significant effect on MSH content ($0\ 88 \pm 0\ 17$ μg/PL mean of 23 animals). In 36 animals the anterior part of the hypothalamus had been destroyed by the lesion in these animals the MSH content amounted to $0\ 69 \pm 0\ 14$ μg/PL ($p < 0\ 05$)

Destruction of the posterior region of the hypothalamus in 15 animals resulted in a MSH content of $1.87 \pm 0\ 40$ μg/PL. The difference with the sham-operated controls was not significant ($p = 0\ 1$)

The results suggest that the hypothalamus may exert a positive as well as a negative influence on the function of the pars intermedia.

REFERENCE

BURGERS, A. C. J Endocrinology 68 698 (1961)

M F D Csánky and D de Wied *Factors involved in the regulation of aldosterone secretion in sodium deficient rats*

Department of Pharmacology Medical Faculty University of Utrecht The Netherlands

Using the rate of aldosterone secretion by rat adrenal tissue *in vitro* as an index of *in vivo* aldosterone secretion, the influence of various factors which might be involved in the regulation of the secretion of aldosterone was investigated in the sodium deficient rat

Aldosterone secretion by adrenals of sodium deficient rats was 2 to 3 times the secretion by adrenals of animals on a standard diet

Six hours after removal of the kidneys from sodium deficient rats the increased rate of aldosterone secretion was not significantly diminished Similarly six hours after total hypophysectomy aldosterone secretion rates *in vitro* were not materially decreased. However removal of both kidneys and hypophysis decreased aldosterone secretion in sodium deficient rats towards the level of that found in animals on a standard diet 6 hours after the operation

Blockade of the CNS by chlorpromazine and nembutal elicited a marked drop in the rate of aldosterone secretion *in vitro* by adrenals of sodium deficient rats within one hour Desoxycorticosterone acetate in a dosage of 2 mg per 100 g injected subcutaneously also decreased aldosterone secretion rates of sodium deficient animals The effect was most marked at 6 hours after injection of this steroid

H J Degenhart* L Frankena H K A Visser* W S Cost** and A P van Seters *Further investigation of a new hereditary defect in the biosynthesis of aldosterone evidence for a defect in 18-hydroxylation of corticosterone*

**Department of Paediatrics State University Hospital 59 Oostersingel Groningen * Red Cross Hospital The Hague **Department of Clinical Endocrinology and Diseases of Metabolism University Hospital Leiden The Netherlands*

Recently we have described three related children with a salt-losing syndrome and urinary C_{21} corticosteroid pattern highly suggestive of an 18-oxidation defect in the biosynthesis of aldosterone (VISSER and COST 1964) Theoretically there are at least two possible defects in the biosynthetic pathway between corticosterone (B) and aldosterone (ALD) In case of a defect in 18-

hydroxylation of B urinary excretion of B metabolites should be high and excretion of tetrahydro-18-hydroxy 11-dehydrocorticosterone (TH 18-OHA, the most important metabolite of 18-OHB) and tetrahydro-aldosterone (TH ALD) should be low In case of a defect of dehydrogenation of the C_{18}-hydroxymethylgroup in 18-OHB urinary excretion of both B metabolites and TH 18-OHA should be high. The latter defect was described by ULICK *et al* (1964)

We have now determined excretion of TH 18-OHA and TH ALD in urine of the two infants from the family described by VISSER and COST (1964) using the methods of ULICK *et al* (1964) with modifications. Urines were collected when infants were 5 and 3½ months old during a period of severe sodium depletion and dehydration. Urine was extracted with CH_2Cl_2 after periodic oxidation. Extracts were purified on silica columns (20 % water <0 03 mm, elution with acetone-chloroform) and chromatography on paper using the systems F/MeCy Tol (1 1) and EtoAc Tol (7 3) SS 589 After acetylation with Ac_2O-C^{14} acetates of etiolactones of TH ALD and TH 18-OHA were chromatographed using the systems F/MeCy $C_2H_4Cl_2$ (SS 289) and $C_2H_4Cl_2$-Tol (1 1) SS 289 Acetates were finally purified by TLC on Kieselgel H (benzene-ether 1 9 $CHCl_3$-MeOH 99 1) and quantitatively estimated using liquid scintillation techniques. TH ALD-H^3 and TH 18-OHA H^3 were isolated from the urine of an adrenalectomized woman after i.v injection of ALD-H^3 and 18-OHB-H^3 Results were

	(µg/24 h urine) TH–ALD	TH 18–OHA
Pat I	2.7	19
Pat II	1.8	27
Control (5/1 yr), basal	19	36
sodium depleted	41	62

These findings strongly suggest an 18-hydroxylation defect as the cause of the salt-losing syndrome

REFERENCES

ULICK, S. *et al.*, J clin. Endocr 24; 669 (1964).
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This work was supported by grants from the Netherlands Organisation for the Advancement of Pure Research (Z W O) We are very thankful to Dr S Ulick (New York) for a gift of tritium labeled 18-hydroxycorticosterone

C A de Groot *Ovarian ascorbic acid depletion in intact and hypophysectomized recipient rats caused by hypothalamic extracts*

Department of Pharmacology University of Leiden The Netherlands

In imitation of McCann we have tried to estimate luteinizing hormone releasing factor (LH RF) with the ovarian ascorbic acid depletion test His method includes preparing acid tissue extracts (from the hypothalamic area containing median eminence and hypophyseal stalk) and heating these for ten minutes in a boiling water bath in order to destroy the protein LH while leaving the less labile peptide LH RF intact

In trying to assess the effectiveness of this form of protein destruction with the aid of crude rat hypophyseal extracts, we found that some LH-activity remained about one eighth of the original amount This remnant of LH-activity is still found after a heating period of 30 minutes A difference with McCann s method is that we neutralize our extracts before injecting them intra venously

We decided to use another way to distinguish between LH and LH RF effects, viz by comparing the ascorbic acid depleting activity of hypothalamic extracts in intact and hypophysectomized recipients against the response of graded doses of NIH LH. The pseudopregnancy of the hypophysectomized female rats was maintained with prolactin

The depleting activity of hypothalamic extracts (heated or not the effects are the same) found in normal rats was often absent in hypophysectomized recipients. A marked activity in hypophysectomized recipients, however can result from hypothalamic extracts obtained from hypophysectomized adult donor rats.

W A den Hartog Jager H J van der Holm J M B V de Jong and M Mator *Studies on plasma creatine phosphokinase in thyroidectomized rabbits*

(To be published in Biochemical Pharmacology)

L G Huis in 't Veld W C de Groot and E B van den Broek *Urinary excretion of stilbene derivatives in cattle after intramuscular injection of Dexerine*

Laboratory of Endocrinology National Institute of Health Amsterdam, The Netherlands

Samples of urine were collected daily from two female 10-week old calves. After one week, one of these animals received an intra muscular injection of 125 mg di-oxy-di-aethylstilbene-di-propionate (jelly form)

The urinary samples were tested for oestrogenic action by a biological technique (Allan Doisy test) and for stilbene derivatives by a chemical technique. The untreated animal showed a very low oestrogen excretion the reaction to stilbene derivatives was negative throughout the investigation in this animal. In the second calf, administration of the synthetic oestrogen was followed by an increase in urinary oestrogen excretion the reaction to stilbene derivatives, moreover was distinctly positive The excretion of oestrogens and stilbene derivatives was determined during a total of 7 weeks.

The results obtained led to the decision to make a large-scale study of the possibility to use urinalysis in testing slaughter-house cattle for having received treatment with oestrogens.

H J van der Molen and K B Eik-Nes *Estimation of total mass and specific radioactivity of several steroids in canine testicular vein blood and testicular tissue following in vivo infusion of 4-^{14}C-3β-hydroxypregn-5-en 20-one via the spermatic artery*

Department of Obstetrics and Gynaecology State University Utrecht The Netherlands and Department of Biological Chemistry School of Medicine University of Utah Salt Lake City Utah, U.S.A

4-^{14}C Pregnenolone with or without ICSH, was infused via the spermatic artery in anesthetized dogs. At several time intervals spermatic venous blood was collected and extracted at the end of the infusion the testes were removed, homogenized and extracted. Following addition of ^{3}H-labeled tracer amounts of steroids the samples were analyzed for both ^{3}H and ^{14}C-labeled and unlabeled androst-5-ene-3β 17β-diol, 3β 17α-dihydroxypregn-5-en

20-one 17α hydroxyprogesterone testosterone dehydroepiandrosterone androst-4-ene-3 17-dione 3β-hydroxypregn 5-en 2-one and progesterone

Separation and identification of the individual steroids was achieved using paper thin layer and gas liquid chromatography Estimation of the mass amounts of the individual steroids was performed following gas liquid chromatography using flame ionization and/or electron capture detection

The results of the estimation of the unlabeled mass amounts of the steroids the accumulation of ^{14}C in individual steroids, as well as the specific radioactivities of the different compounds were discussed in the light of the existing concepts of steroid biosynthesis in the (dog) testis.

G P van Rees *Anterior-pituitary FSH and LH levels before, during and after pseudo-pregnancy in the rat*

(To be published in Acta Endocrinologica)

P G Smelik *A dopaminergic innervation of the intermediate lobe of the pituitary?*

Department of Pharmacology Medical Faculty University of Utrecht The Netherlands

A histochemical method for the detection of monoamines according to Falck was applied to hypothalamic and hypophyseal tissue of albino rats An intense fluorescence indicating the presence of noradrenaline or dopamine was found in the median eminence the stalk and the intermediate lobe The morphological appearance suggests that fibre tracts originating in the median eminence enter the intermediate lobe via the hypophyseal stalk.

In order to differentiate whether the fluorescent material is derived from noradrenaline or from dopamine several pharmacological pretreatments were used. Administration of reserpine caused a complete disappearance of the fluorescent material. Pretreatment with α methyl meta tyrosine which converts noradrenaline into non-fluorescent metaraminol did not alter the fluorescence Injection of α methyl-dopa followed by administration of reserpine by which procedure only dopamine is depleted, resulted in a depletion of fluorescent material Finally disulfiram treatment

blocking the conversion from dopamine into noradrenaline had no effect on the fluorescent material.

It is concluded that the median eminence-stalk region and the intermediate lobe contain a high dopamine content suggesting that dopaminergic pathways of central origin to the intermediate lobe exist, which may play a role in the endocrine function of the intermediate lobe

T Tjabbes J D Baars and P S Blom *Glucose-6-phosphate dehydrogenase (G-6-PD) determinations in human adipose tissue*

The Municipal Hospitals The Hague The Netherlands

The content of G-6-PD was determined in human subcutaneous- and omental adipose tissue The adipose tissue was obtained during operation the age of the persons ranged from 23 to 84. The G-6-PD content of adipose tissue is of great importance for the activity of the hexose-monophosphate shunt. The NADPH, caused by this way is essential for the appearance of long-chain fatty acids so the G-6-PD activity has an influence on the fatty acid synthesis.

The G-6-PD activity in the omental adipose tissue was significantly higher than in the subcutaneous adipose tissue. There was found no difference of the G-6-PD content of adipose tissues between men and women. The G-6-PD activity of the obese persons was lower than of normal and lean persons. This difference was only significant for the omental adipose tissue.

It was concluded that by this way we found no difference between the fatty acid synthesis of men and women also we found no evidence for a higher fatty acid synthesis in adipose tissue of the obese

A Vermeulen *Determination of production rate of some hormonal steroids*

Department of Internal Medicine University of Gent Belgium

Methods are based either on urinary isotope dilution or on metabolic clearance rate (M.C.R.).

Principle of isotope dilution a radioactive hormone tracer introduced in the blood stream will be metabolized just like the endogenous hormone all metabolites of the endogenous hormone will be labelled and the dilution of the tracer is a measure of the quantity of

hormone secreted The metabolic clearance rate can be defined as the volume of plasma cleared completely and irreversibly of the hormone per unit of time In conditions of steady state the hormone production rate is then M C R × plasma concentration of the hormone

In the absence of a unique urinary metabolite of the hormone the urinary isotope dilution method cannot be used the metabolic clearance rate on the other hand gives only meaningful results if plasma hormone levels can be accurately determined and if the latter do not show important nycthemeral variations In the latter cases hourly production rates can be determined but these may not be extrapolated to the 24 h

Cortisol production rate is generally determined by the isotope dilution method Best results are obtained when after initial separation by paper chromatography H_4E and H_4F are oxidized with periodic acid (Metcalf Fox) and the 11 OH-etiocholanolone is chromatographed on thin layer Final colorimetry is performed with the Zimmermann reaction Normal production values 15–30 mg/24 h

Corticosterone production rate is determined by measuring the specific activity of H_4B and allo H_4B using B T for colorimetry the low specificity of the latter reaction requires extensive purification of the extract Normal values 0 5–4 mg/24 h

Compound S production is determined by oxidation of H_4S isolated after initial paper chromatography Direct oxidation in the urine leads to falsely high values as pregnanetriol is also oxidized to etiocholanolone Normal values 0 2–1 mg/24 h

Aldosterone production rates can be determined using either the aldosterone-3-oxo-conjugate or *tetrahydro-aldosterone* When the 3-oxo-conjugate is used double isotope techniques are generally employed Tetrahydro-aldosterone allows the use of blue tetrazolium for quantitation although here again careful purification is required due to the low specificity of B T Normal values 50–°00 µg/24 h

The use of the isotope dilution technique for the determination of *testosterone production* is subject to much criticism at least when used in females or hypogonadal males Indeed testosterone glucuronide used to determine the isotope dilution cannot be considered to be a unique metabolite of testosterone but arises for an ap-

preciable amount directly from androstenedione in the peripheral tissues without testosterone being secreted in the blood stream. With the increased sensitivity of methods for the determination of plasma testosterone the M.C.R. method seems to be the method of choice for the determination of testosterone production. Normal values males 4–14 mg/24 h, females 0 3–1 5 mg/24 h.

J van der Vies and J de Visser *The action and excretion of oestriol dihemisuccinate after intravenous administration*

Endocrinological Research Department N V Organon, Oss The Netherlands

The half life time of oestriol dihemisuccinate in blood after intravenous administration to patients was found to be 7.2 min, which is in agreement with the results obtained in rats in previous experiments. The urinary excretion of oestrogens was increased for a period of several days following intravenous injection of the steroid to patients, indicating a slow elimination from the body This long lasting excretion was rather unexpected because clearing from the blood occurs so rapidly Experiments on rats were under taken in order to prove whether the long lasting urinary excretion of oestrogens represented a biologically active level in the body

B van der Wal J Bruinvels M F D Csánky and D de Wied *Effect of ACTH and angiotensin II on aldosterone secretion by rat adrenal tissue in vitro*

Department of Pharmacology Medical Faculty University of Utrecht The Netherlands

Total corticosteroid and aldosterone secretion by rat adrenal glands *in vitro* were used as an index of *in vivo* adrenocortical activity

Quartered adrenal glands were incubated for 1 or hours under suitable conditions. Total corticosteroid secretion was determined by U V absorption. Aldosterone was isolated by thin layer chromatography and measured by sodium fluorescence on paper

The effect of ACTH and angiotensin II on aldosterone secretion was studied in rats on standard diet and on Na-deficient diet

In rats on standard diet the level of endogenous ACTH was altered by noxious stimuli and hypophysectomy Whereas a marked

influence on total corticosteroid secretion was observed aldosterone secretion remained at the level of control animals. The injection of a high dose of long-acting ACTH into hypophysectomized animals caused a slight but non-significant increase in aldosterone secretion β Angiotensin was administered to intact and hypophysectomized rats. No effect on aldosterone secretion could be demonstrated

In Na-deficient animals however aldosterone secretion was markedly increased by the injection of ACTH It appeared that in these rats exogenous as well as endogenous ACTH stimulates aldosterone secretion considerably No increase in aldosterone secretion was observed when angiotensin II was administered to Na-deficient hypophysectomized rats.

G H Zeilmaker *Prolonged lactation in mice and its effect on mammary tumorigenesis*

Biological Department The Netherlands Cancer Institute Amsterdam

In previous studies lactation in mice could be maintained during two months by litter replacement (PARKES 1926 SELYE and McKEOWN 1934 MÜHLBOCK and TENGBERGEN 1961) In 3 multi parous rats BRUCH (1958 1961) could prolong lactation during 10–12 months by providing them with 8–10 days old litters.

In an attempt to explore the effects of prolonged lactation on mammary gland tumorigenesis 34 ($C_3H_f \times O_{20}$) F_1 hybrid mice received 2 pituitary isografts under the kidney capsule during pregnancy The lactation period in 27 of these animals could be prolonged to 7 months by giving them 0–2 days old infant mice twice weekly The average combined weight gain of the two litters was 10 grams per week In 7 otherwise identical cases milk production ceased after 4–6 months.

In 13 mice without pituitary grafts the lactation performance gradually declined from 30 g litter growth per week to 5–10 g per week over a period of two months. Following exposure to older young an increase in milk yield was obtained (5–10 g litter growth per week after 7 months) in 11 cases.

In a third experiment 10 females bearing 2 pituitary isografts were unilaterally thelectomized Five animals were allowed to nurse the others served as controls. After two months of lactation

the suckled glands were actively secreting milk while in the blocked glands the alveoli had atrophied (WILLIAMS 1941) except for those localized in the periphery of the gland. These peripheral alveoli had probably been formed recently and would have regressed later after a period of distension. In the non-suckled controls the alveolar apparatus in both the intact and thelectomized glands was less well preserved than in the thelectomized glands of the suckled mice. Many hyperplastic 'nodules' were observed in the glands of the non-suckled mice.

The difference in maintenance of the alveolar system in the thelectomized glands of the suckled and non-suckled mice can probably be attributed to the function of the pituitary gland *in situ*. This would imply that the mammotrophic secretions of the pituitary gland *in situ* of the suckled mice augment significantly to the effect of the LTH secreted by the two pituitary grafts.

In a fourth experiment the effect of prolonged lactation on mammary tumorigenesis was studied in MTV bearing $(C_3H \times O_{20})F_1$ hybrids, which all had received 2 pituitary isografts during the latter half of pregnancy. Eleven of these mice were allowed to nurse continuously following parturition. The other 11 animals were not allowed to nurse. Ten of these had developed 16 mammary tumors within 6 months post partum. The earliest tumor appeared 3½ months post partum.

In only one of the lactating animals a tumor occurred so far the post partum period being 6½ months.

This experiment in which the secondary hormonal effects of the suckling stimulus and a possible aspecific noduligenic effect of unilateral thelectomy (FEKETE and LITTLE, 1952) were eliminated, clearly shows that lactation prevents tumorigenesis to a large extent.

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XXIV INTERNATIONAL CONGRESS OF PHYSIOLOGICAL SCIENCES

The XXIV International Congress of Physiological Sciences will be held in Washington D C U.S.A August 25–30 1968 The Congress is sponsored by the International Union of Physiological Sciences (IUPS)

Preliminary notices will be mailed in January 1967 and final notices in October 1967 Plans are already being made for special symposia and invited speakers. Specific suggestions for symposium topics or special lectures should be submitted as early as possible to the President of the Congress Professor Wallace O Fenn University of Rochester Medical Center Rochester New York 14620 U S A

In selecting topics for symposia it is expected that the Program Committee will give preference to subjects of a somewhat controversial nature but of broad general interest and not recently covered in an international symposium For speakers special consideration should be given to promising young physiologists with active research programs as well as to older men of established reputation

All inquiries concerning the Congress may be addressed to the Congress Manager Mrs Helena B Lemp XXIV International Congress of Physiological Sciences, 9650 Rockville Pike Bethesda, Maryland 20014 U.S.A

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Department of Physiology, University of Nijmegen

MEAN BODY TEMPERATURE AND THE CONTROL OF THERMAL SWEATING

BY

J. W. SNELLEN

CONTENTS

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In selecting topics for symposia it is expected that the Program Committee will give preference to subjects of a somewhat controversial nature but of broad general interest and not recently covered in an international symposium For speakers special consideration should be given to promising young physiologists with active research programs as well as to older men of established reputation

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1 INTRODUCTION

1 1 General introduction

A man in a moderately warm and dry environment sweats in order to lose a required amount of calories provided that the sweat producing mechanism can meet the demands. For example at a work load of 7 0 kcal/min in an ambient temperature such that the heat loss by radiation, convection and conduction, i.e the non-evaporative heat exchange, is 2.0 kcal/min, the man sweats after a certain time lag in order to dissipate 5 0 kcal/min. This maintenance of thermal equilibrium is, as Glaser and Newling (1957) pointed out, the fundamental property of temperature control in man. This is, however a truism since it only means that the first law of thermodynamics known in this field of physiology as the heat balance equation is valid when applied to a temperature controlled system a thermostatically controlled water bath also shows a perfectly maintained thermal balance albeit usually with an on-off type of control. The observation of a heat balance does not give any information as to how and on what input *the control mechanism acts it only states that the control mechanism* works properly

The problems of thermoregulation in humans arise in the search for the appropriate incoming signal of the regulatory mechanism. Many investigators agree that there is some interaction between internal (rectal oesophageal tympanic) and superficial (skin) temperatures as the driving force for thermal sweating. Great

Possibly the best solution for the problems of the mode of interaction between internal and superficial temperatures is the one given by HARDY (1961) it is a type of summation of the combined inputs from the(se) receptors all over the body surface and in other body tissues that finally determines the action of the temperature regulating system" According to this, other thermoreceptors than those in the hypothalamus (NAKAYAMA, 1961 HARDY *et al* 1964) and the skin as has been demonstrated in the spinal cord (THAUER, 1962) and respiratory tract (BLIGH 1957) can also play a role It agrees with VAN BEAUMONT's statement that "the measurement of temperatures at other sites than rectum tympanic membrane and epidermis warrants serious consideration"

The present is an attempt to estimate the change in mean body temperature during different combinations of work and heat load or in other words "the summation of the combined inputs from the receptors all over the body" from direct calorimetrical measurements instead of the usual procedure of weighting skin and internal temperatures. It seems worthwhile in many respects to combine calorimetry and thermometry not only because this provides a necessary cross check but also because it might shed new light on experimental facts which have so far not fitted into the concept of thermoregulation, such as

1 1 1

After a period of work and heat load the sweating rate drops very rapidly in fact much faster than any internal temperature. Very few time constants are reported explicitly in the literature. From a graph published by NIELSEN and NIELSEN (1962) a time constant of 4 5 min can be estimated for deep esophageal temperature WENZEL (1964) reports a double exponential function for the decline of rectal temperature with time after exercise where the time constants are 3 7 and 7 0 min respectively ROBINSON (1963) reports that sweating in man drops precipitously and as fast as the temperature of the blood in the femoral vein, while the muscle temperature lags behind. In his recent study (ROBINSON *et al* 1965) quite a few graphs are given representing sweating rates and a number of usual temperatures and ones rarely investi

controversy exists however as to the problem how this interaction takes place. Those investigators who base their conclusions largely on steady state or quasi steady state observations find a type of interaction first proposed by ROBINSON (1949): a more or less straight line relationship between internal temperature (on the abscissa) and sweating rate (on the ordinate) at a high mean skin temperature, this relation being shifted to the right at lower mean skin temperatures. ROBINSON and also NIELSEN and NIELSEN (1965) find nearly straight lines, BENZINGER *et al.* (1963) slightly curved lines, WYNDHAM (1965) a highly non-linear curve (saturation of the control system) with smaller inclinations when shifted to the right. BENZINGER rejects a shift to the left at skin temperatures higher than 33° C, whereas WYNDHAM demonstrates such a shift. These investigators agree that internal temperature is mainly determined by the metabolic rate and is independent over a wide range of ambient temperatures, and that mean skin temperature is mainly determined by ambient conditions and largely independent of metabolic and/or sweating rate (NIELSEN 1938). Other investigators who measure fast responses point out that the above mentioned type of interaction cannot account for peripheral reflex sweating. BREBNER and McK. KERSLAKE (1961) demonstrate that the forearm sweating rate follows the cycles of cyclic radiant heating of the trunk with a delay of not more than 1.0 sec. In his extensive review of the present state of knowledge in this field VAN BEAUMONT (1965) demonstrates a delay of not more than 1.5 sec after starting exercise. GAGGE (1964) confirms the rapid response of the sudomotor system to radiant heat; this response is even approximately quantitative: a gain of 130 kcal/m² h was counteracted by an increase in evaporation of 100 kcal/m² h within two min. The associated rise in skin temperature was just over 2° C, accounting for an additional heat loss of perhaps some 15 kcal/m² h. A similar quantitative relationship between heat gain and heat loss is demonstrated in another field of thermoregulation, namely in vasomotor reflexes. COOPER *et al.* show a quantitative relationship between heat input by radiation (1950) or immersion of an arm in hot water (1964) and heat loss from the finger with astonishingly short delay times, both in febrile and afebrile patients, provided that the internal (oral) temperature is stable at the elevated level.

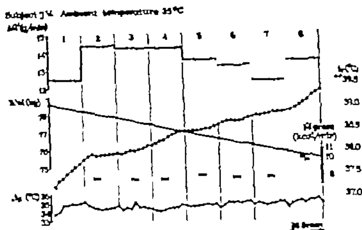


Fig. 1.1

Prolonged heavy muscular exercise (8 kcal/min) during nearly 4 h. In the last 28.8 min the work load was set higher (10 kcal/min) to demonstrate that the sweat producing mechanism is capable of increasing its rate. Calorimetric analysis see Table 4 1. $\bar{t}_s$ mean skin temperature, not corrected for the systematic error of the measuring device; M_{gross} metabolic rate; BW: body weight; $\Delta G'$ weight loss due to evaporation.

sary to assume that the set point is reset at a higher value under certain conditions. At present fever is recognized as the result of such a set point shift (COOPER *et al* 1964 ANDERSEN *et al* 1961). The rise in internal temperature due to exercise is also explained by an elevation of the set point temperature (NIELSEN and NIELSEN 1962) although JACKSON and HAMMEL (1963) point out that this has never been definitely proven. They investigated hypothalamic temperatures in exercising dogs and found no rise in this temperature.

1.2 PLAN OF INVESTIGATION

A combination of thermometry and calorimetry means that besides the current body temperatures (rectal, skin and mean skin) all left hand terms of the heat balance equation, i.e. heat production by metabolism, heat loss by evaporation and non-evaporative heat exchange must be measured as continuously and directly as possible in kcal/min or other units of power An estimate of the amount of heat accumulated in the body during the period of

gated (such as muscle saphenous and femoral vein temperatures) against time

1 1 2

SNELLEN (1960) demonstrated by means of the heat balance equation that external work in grade walking on the treadmill was identical with the caloric equivalent of body weight times gained height A striking phenomenon although not mentioned in that paper can be seen in figure one of that study *namely that* during one experiment while gross metabolic rate heat exchange by radiation and convection body temperatures and environmental conditions were *essentially* unaltered the *sweating rate and in* consequence (in this case) the heat loss by evaporation changed *by exactly the same amount as the caloric equivalent of the* external work.

1 1 3

It is known that during prolonged exercise in a hot environment without drinking the rectal temperature shows a small but constant rise after reaching the level corresponding to the metabolic rate (PITTS *et al* 1944) For the sake of demonstration such an experiment has been repeated with the tools described in Chapter 2 and this is shown in Fig 1 1 The rectal temperature will reach a constant level only when the subject is allowed to drink (PITTS *et al* 1944 MACPHERSON 1960) Moreover when the subject is hyperhydrated the rectal temperature will reach a lower level than the one corresponding to the metabolic rate (MOROFF and BASS 1965) *The divergence between sweating rate and body temperature as shown in Fig 1 1 cannot be explained by the current concept of thermoregulation*

The problems mentioned above might all be covered by a model in which some temperature which is vaguely a summation of the combined inputs is compared with a set point value and in which the difference from this set point value acts as the stimulus for the sweat producing mechanism Maintenance of the balance between heat input and output can be achieved merely by sensing a temperature However several observations have made it neces-

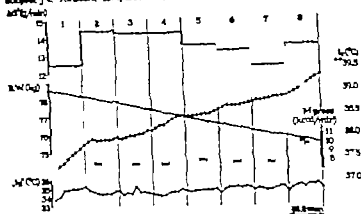


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work and heat load and dissipated in the recovery period can be made from the algebraic sum of the total amounts of calories gained (and/or produced) and lost in each period provided that at the start and the end of each period gains and losses are in equilibrium

It is desirable that the subject starts each experiment from as nearly as possible identical conditions and it is imperative for the above mentioned estimate of the accumulated heat during the period of work and heat load that he reaches a thermal steady state prior to the period of work and heat load

1 3 Experimental design

From the above follows the experimental procedure namely

a) a period of sitting in a given climate until all relevant physiological variables (rectal and skin temperatures non-evaporative heat exchange) and responses (metabolism sweating rate) reach a constant level

b) a period of exposure to a combination of work and heat load until a thermal steady state is reached

c) a period of recovery sitting in the climate as under a) until the above mentioned physiological variables and responses return to their original levels.

During the whole procedure rectal and skin temperatures as well as air temperatures and humidity are measured at small time intervals During a) and c) weight loss and non-evaporative heat exchange are measured continuously and metabolic heat production at appropriate times During b) weight loss is measured at intervals and metabolic heat production is measured over a convenient period of time This procedure is repeated starting from several initial climatic conditions and with different combinations of work and heat load

2 SUBJECTS AND METHODS

2 1 Subjects

Two healthy male students served as subjects. Their physical characteristics were

	subject W.K.	subject v T
age	22 years	23 years
body height in m	1 78	1 76
body weight in kg	76.83 s.d. 0.285 (1st series)	74.06 s.d. 0.664
	79.54 s.d. 0.823 (2nd series)	
body surface area	1.96 m²	1.90 m
(Dubois and Dubois, 1916)		

Subject W K was a medical student who was not specially trained for physical exertion. Between the first and second series of experiments his body weight increased by approx. 4 kg which he gradually lost again during the second series of experiments. Subject v T was a student in physical education who was, thanks to his education, well trained to overcome physical exertion Both subjects were not acclimatised to heat. During the experiments they were clad in shorts socks and gymnasium shoes. They were not allowed to drink during this period.

2.2. Sequence of the experiments

2.2.1

To test the plan outlined above a series of experiments was carried out with subject W.K. according to the schedule given in Fig. 2 1 The exposure times were chosen according to previous

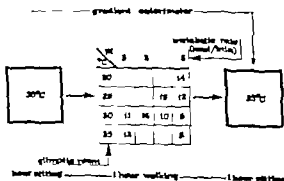


Fig. 2.1

Schedule of the first series of experiments with subject W.K., starting from and ending t gradient-calorimeter temperature of 25° C. The figures in the central block diagram giv the experiment numbers in chronological order

experience preliminary experiments with the gradient calorimeter and the harness (see 2 3 2 3) have shown that one hour for the first period of sitting was sufficient to reach a balance between heat gains and heat losses three weighing intervals in one hour were sufficient to obtain a duplicate determination of weight loss the duration of the second period of sitting was one hour mostly owing to limitations in available time The figures in the central block diagram represent the experiment numbers in chronological order They alternated light-heavy light heavy according to previous experience using sweating rate as a measure There was one experiment a day and not more than two a week. This series of 16 determinations gave *promising results* (see e.g middle graph subject W K Fig 3 27)

2 2 2

A larger series was designed for a second subject v T with three calorimeter temperatures and 12 combinations of work and heat load per calorimeter temperature or 36 experiments in total. The sequence of the experiments was randomised by ranking them in order of severity and ranking 36 consecutive numbers in a table of random numbers. The result is given in Fig 2 2 This series of experiments with the second subject could not be completed due to an injury to his foot (not occurring during the experiments) After his recovery the remaining available days were so used that as much as possible the same combinations of work and heat load per calorimeter temperature were investigated 26 experiments in total were made on subject v T

2 2 3

The first subject completed also two series of experiments starting from the two other gradient-calorimeter temperatures The sequence of his experiments was taken from that of the other subject omitting all experiments starting with 35 C A total of 40 experiments were made on this subject. His complete schedule is given in Fig 2 3

Where necessary a given experimental procedure *is abbreviated* as e g 35–7½–20 indicating a gradient-calorimeter temperature of

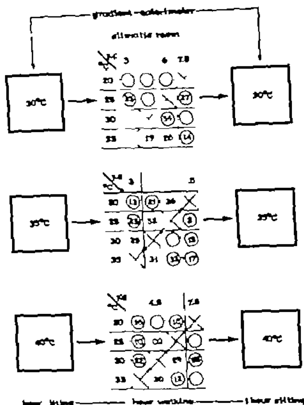


Fig. 2.2.

Schedule of the series of experiments designed for subject v T. The ringed experiments were actually carried out. Number of experiments 26

35° C a net metabolic heat production of 7½ kcal/min in the climatic room at 20° C.

2.3. MEASUREMENT OF BODY TEMPERATURES

The same principle was applied throughout: the rectal temperature was measured with a thermistor and all other body temperatures were measured with copper-constantan thermocouples as differences against the rectal temperature so the reference temperature might change by some degrees. A difference, however of e.g. 10° C with a reference temperature of 36° C gives almost the same E.M.F. as a difference of 10° C with 38° C as a reference temperature (Handbook of Chemistry and Physics p. 2717, 1961).

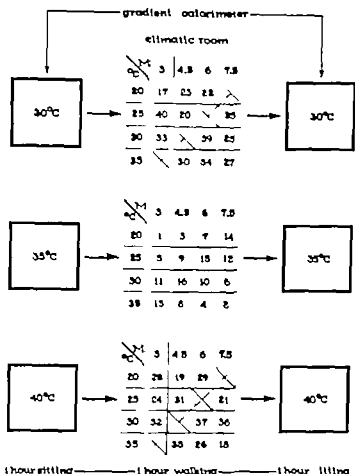


Fig 3.

Schedule of the complete series for subject W K Number of experiments 40

2 3 1 *Rectal temperature*

The rectal temperature was measured at a distance of 20 cm from the sphincter In the metal tip of the rectal element (11 mm diameter) the thermistor mounted on a high quality co-axial cable and 20 copper-constantan thermojunctions were embedded in artificial resin thus electrically isolated from each other and from the subject Calibration of the thermistor including the extension cable was carried out with the rectal element placed in an oil filled thermos flask mounted in a thermostatically controlled oil bath against a 0 02 C scale division calibrated thermometer The resistance of the thermistor was measured with a 5 decade Wheatstone bridge fed by a 1 5 V dry battery with 12 kΩ in series

and a Kipp AL2 galvanometer as a zero indicator The thermometer readings corrected with the emergent stem correction (Handbook of Chemistry and Physics p 2418 1961) and the logarithm of the resistance showed a slightly curved line in the range of 35° C and 40° C but assuming a straight line in this range the error was less than 0 01 C. The effect of ageing is a disadvantage in using thermistors. Before each series of experiments the calibration was repeated. The time constant of the thermistor in this rectal element was 2.5 min.

2.3 2. *Skin temperatures and mean skin temperature*

Twenty four thermojunctions were distributed over the body surface as shown in Fig 2.4. Their distribution and their weighting

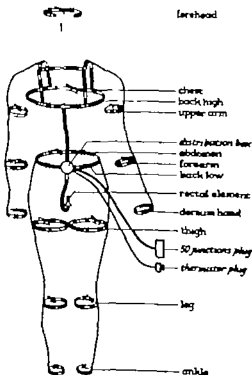


Fig 2.4

Schematic drawing of the "harness" seen from behind, and the distribution of the skin thermojunctions.

factors were more or less according to HARDY and DUBOIS (1939) Table 2.1. With twelve 4 switch relays the 24 thermal E.M.F.s

TABLE 2.1

Weighting factors for the skin thermocouples

Hardy and Du Bois		place of thermocoupl		weight fact	point nr recorder
head	0.07	forehead	right	0.035	1
		forehead	left	0.035	
		chest	right	0.04375	3
		chest	left	0.04375	
		back high	right	0.04375	5
trunk	0.35	back high	left	0.043 5	
		abdomen	right	0.043 5	7
		abdomen	left	0.043 5	
		back low	right	0.04375	9
		back low	left	0.043 5	
		right upperarm ventral		0.04	11
arms	0.14	right upperarm dorsal		0.04	
		left forearm	ventral	0.03	13
		left forearm	dorsal	0.03	
hand	0.05	dorsum right hand		0.0*5	15
		dorsum left hand		0.0*5	
		right thigh	ventral	0.0475	17
thighs	0.19	right thigh	dorsal	0.04 5	
		left thigh	ventral	0.0475	19
		left thigh	dorsal	0.0475	
		right shin		0.065	1
legs	0.*0	right calf		0.065	
		right ankle		0.035	*3
		left ankle		0.035	

were either added two by two mostly from symmetrical parts of the body or presented to a voltage divider and averaged electrically taking into account the weighting factors. A schematic wiring diagram of two thermocouples is given in Fig. . 5. A 24 point recorder (1 mV full scale deflection) records the 1. pairs of E.M.F.s once and the mean E.M.F. twice in one cycle (. min). In each

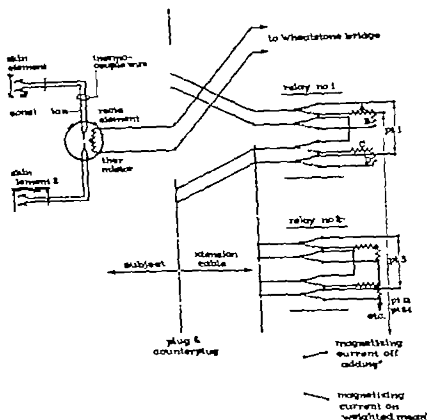


Fig. 2.5.

Schematic wiring diagram of two out of the 24 thermocouples.

skin element both the copper and constantan wire were soldered to multicore plastic covered copper wires. The junctions were embedded in artificial resin forming an element of approx. $0.3 \times 1 \times 2$ cm. These elements were sandwiched between two layers of elastic textile band from which the "harness" was made. All wires to and from the skin elements ran through plastic tubing sewn to the "harness". The resistances of the voltage dividers were calculated individually. The sum of the parallel resistances B and D (see Fig. 2.5) in all twelve relays should not exceed 500 Ω to match the input impedance of the recorder. The individual parallel resistances were made of thin copper wire of approx. 20 Ω each

and wound on a glass rod The series resistances A and C in all twelve relays were calculated and manufactured accordingly taking into account

1) the actual resistance of the parallel resistor R_p
2) the internal resistance of the thermocouple including the extension wire R_i
3) the fraction of the total body surface area the temperature of which the thermocouple is supposed to represent p
4) a factor 2 since the recorder is printing the individual temperature differences on a double scale (two E.M.F s added)

In an equation
$$\frac{R_p}{R_i + R_p + x} = \frac{2p}{100}$$

The calibration consists of 2 3 2 1 the determination of the relation between temperature difference (Δt) and E.M F 2 3 2 2 testing the validity of the weighted mean temperature difference as calculated electrically and 2 3 2 3 testing the validity of the mean skin temperature as measured with this harness and this type of skin elements An evaluation of the validity of one single skin temperature will be omitted in this study

2 3 2 1

All calibrations were carried out with the extension cable in position The skin elements were bundled together as closely as possible and put in a container with reasonable thermal isolation and standing in a temperature controlled room The temperature in the container was read off from a 0 02 C scale division calibrated thermometer Temperature differences were achieved by varying the room temperature and/or the rectal element temperature Readings were taken once a day The following results were obtained for pairs of individual thermocouples

$$y = 0\ 1158\ x - 0\ 02 \qquad \text{s d}_{\text{diff}} = 0\ 03$$

for the electrically calculated average

$$y = 0\ 1205\ x - 0\ 03$$

where y = temperature difference (Δt)
x = scale units (U)

The electrical zero was set at $U = 1.0$ The sensitivity of the pairs of thermocouples (1000/100)/(2 0 1168) = 43μV/°C, 100 units $\hat{=}$ 1 mV) is slightly higher than that of the electrical averager (41 μV/°C). The ratio is 1 0406

2.3.2.2.

In actual experiments skin temperatures differ regionally specially in cool environments. In Fig 2 6 a comparison has been

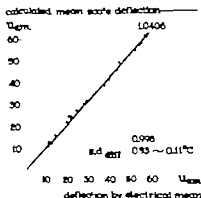


Fig. 2.6.

Comparison of calculated weighted mean deflection of the recorder with observed deflection caused by the electrical mean. The line is not the regression line but a line with regression coefficient 1.041 (see 2.3.2.1).

made using data from such experiments, between the deflection of the recorder caused by the electrical averager (electrical mean) and the weighted mean deflection (calculated mean) computed from

$$U_{cm} = U_1 \; 2p_1 + U_2 \;\; p_2 - - - - + U_{23} \; 2p_{23}$$

where U_1 = deflection of point 1

p_1 = twice the weighting factor of point 1 (Table 2.1).

As an example the values of the highest and the lowest point in Fig 2.6 are given in Table 2.2. The standard deviation of the differences is 0.93 scale units, which equals 0.11 °C.

TABLE 2.2

Figures from which the highest and the lowest point in Fig 0 are calculated

point	1	3	5	7	9	11	elec. mean 1	13	15	17	19	21	..3	calc mean
low	11 6	10.3	11 8	13 8	10 7	1 4	11..	3.3	7.2	15.4	13.1	15.5	9.5	12.4
high	41 °	40 6	50 0	47 8	53.5	56.5	56 °	61 °	78 °	7 0	09..	65.8	43.1	57.5

2 3 2 3

Whether any device for measuring skin temperatures is really indicating the true skin temperature is a never ending argument, as BENZINGER (1959) states. Contrary to his technique of validation (which is essentially not more than a check on the zero instead of a final settlement of the argument) in this study the non evaporative heat exchange has been plotted against the difference between air temperature and weighted mean skin temperature of the skin thermojunctions The non-evaporative heat exchange was measured with the gradient calorimeter described below In Fig 2 7 the results of a calibration on three subjects are shown

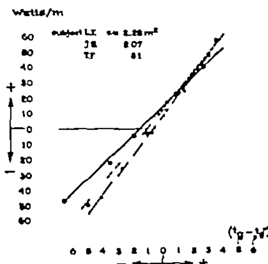


Fig 2 7

Non-evaporative heat exchange in Watts/m² of three subjects, pl tted against the differences between air temperature (t) and mean temperature of the thermojunctions (t)

The calibration lines are straight and do not pass through the origin. It is noteworthy that the "harness" measures too high temperatures. When the non-evaporative heat exchange is zero the difference between air (and wall) temperatures (t_a) and weighted mean skin temperature should be zero too. The deviation from the zero is constant for each individual subject as seen from Fig. 2.7. Moreover there is an influence of body size on the mean temperature of the thermojunctions, the error being larger in obese subjects. This means that the "harness" has to be calibrated for each subject. An explanation why the "harness" is measuring too high temperatures is highly speculative as long as model experiments are not carried out. For this study the empirical calibration line and the subsequent correction constant are sufficient to calculate true mean skin temperature.

2.4 GRADIENT CALORIMETER

The name of this instrument is erroneous. It is not a calorimeter in the strict sense nor does it measure heat flux over a given isolation by measuring the gradient over this isolation. The principle is the measurement of the electrical energy required to maintain a chosen thermal gradient between inside and outside with and without an extra heat source (heat dissipation of the subject) or heat sink (heat absorption by the subject). Since no suitable word could be found the apparatus will be called gradient calorimeter. The apparatus (Fig. 2.8) is a double walled wooden box with inner dimensions of 130 × 100 × 90 cm, ventilated with approx. 450 l/min; the air is "stirred" with a second blower mounted inside. A network of 674 thermocouples, connected in series, was mounted over all six walls (Fig. 2.9). The soldered junctions protruded in the air except for the floor inside where they were pressed into the wood. From the last thermocouple the return wire ran back to the first along the external thermojunctions in order to avoid picking up A.C. hum. The box was heated with two electrical heating elements (250 W each) mounted in the air stream of the ventilator. One heater was connected with a variable transformer mounted on the top of the box. It is set once as required before the experiment starts (permanent heat input). The other was fed by a motor driven variable transformer. The driving motor was part of a Honeywell-

Fig. 8.
Gradient calorimeter mounted on the balance

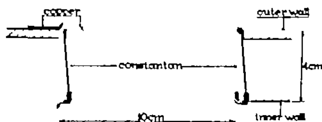


Fig 9
Schematic wiring diagram of two out of the 674 thermocouples.

Brown continuous balance system controlling the thermal gradient over the walls of the box The electrical energy of this heater was detected by a thermocouple element normally used in antenna circuits of radio emitters and recorded on the 24 point recorder

six times per cycle (7.2 min) The connecting wires to and from the door ran over the hinges in spiral loops. After some training it was possible to counteract satisfactorily the disturbing effect of opening and closing the door by switching on an extra 500 W heating element for some seconds. The gradient calorimeter was placed in a room with controlled temperature (range *0–34 C, accuracy 0.5 C reproducibility approx. 1 C) Keeping the room temperature and the gradient constant a stable inner temperature was obtained to which the subject was exposed. The gradient was controlled by comparing the thermal E.M.F from the network of thermocouples with a reference voltage and feeding this voltage difference into the Honeywell Brown chopper amplifier (Fig 2.10) In order to obtain a stable system the output of the amplifier was differentiated with a small D C. motor on the axis of the transmission between servomotor and variable transformer acting as

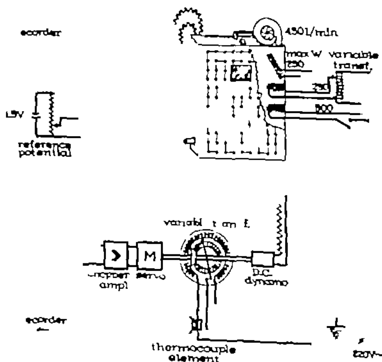


Fig. 2.10

Schematic diagram of the control mechanism of the thermal gradient.

Fig 2.8

Gradient calorimeter mounted on the balance.

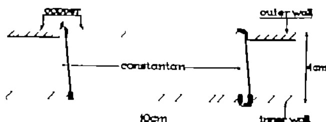


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six times per cycle (7.2 min). The connecting wires to and from the door ran over the hinges in spiral loops. After some training it was possible to counteract satisfactorily the disturbing effect of opening and closing the door by switching on an extra 500 W heating element for some seconds. The gradient calorimeter was placed in a room with controlled temperature (range 20–34 C, accuracy 0.5° C reproducibility approx. 1 C). Keeping the room temperature and the gradient constant a stable inner temperature was obtained to which the subject was exposed. The gradient was controlled by comparing the thermal E.M.F. from the network of thermocouples with a reference voltage and feeding this voltage difference into the Honeywell Brown chopper amplifier (Fig. 2.10). In order to obtain a stable system the output of the amplifier was differentuated with a small D.C. motor on the axis of the transmission between servomotor and variable transformer acting as

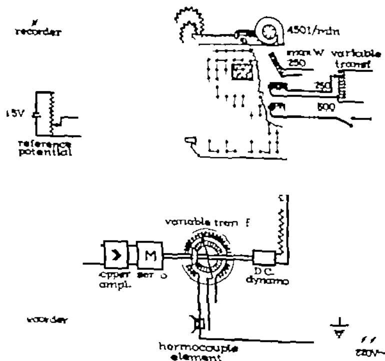


Fig. 2.10.

Schematic diagram of the control mechanism of the thermal gradient.

Fig 8.

Gradient calorimeter mounted on the balance

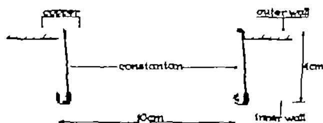


Fig 9

Schematic wiring diagram of two out of the 674 thermocouples.

Brown continuous balance system controlling the thermal gradient over the walls of the box The electrical energy of this heater was detected by a thermocouple element, normally used in antenna circuits of radio emitters and recorded on the 24 point recorder

six times per cycle (7.2 min) The connecting wires to and from the door ran over the hinges in spiral loops. After some training it was possible to counteract satisfactorily the disturbing effect of opening and closing the door by switching on an extra 500 W heating element for some seconds. The gradient calorimeter was placed in a room with controlled temperature (range 20–34 C, accuracy 0.5° C reproducibility approx. 1 C). Keeping the room temperature and the gradient constant a stable inner temperature was obtained to which the subject was exposed The gradient was controlled by comparing the thermal E.M.F from the network of thermocouples with a reference voltage and feeding this voltage difference into the Honeywell Brown chopper amplifier (Fig .10) In order to obtain a stable system the output of the amplifier was differentiated with a small D C. motor on the axis of the transmission between servomotor and variable transformer acting as

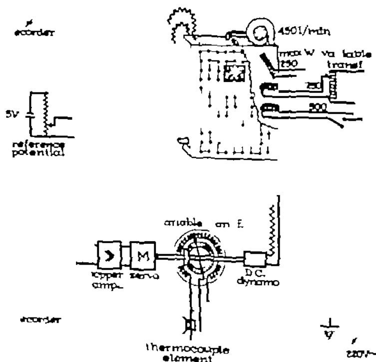


Fig. 2.10.

Schematic diagram of the control mechanism of the thermal gradient.

a dynamo which added its voltage with reversed sign to the input signal In the gradient calorimeter a garden chair was placed with plastic cords for the seat and back support An adjustable head support enabled the subject to sit quietly and completely relaxed. A small 25 W bulb served for illumination A respiration valve adjustable for position hung from the ceiling Since the ventilator made some overpressure in the box sufficient to lift the membranes of the valve both inlet and outlet of the valve were connected to tubes perforating the ceiling The subject inspired room air instead of calorimeter air and expired into the room or a Douglas bag This heat loss from the system was assumed to be negligible The calibration of the gradient calorimeter included

a) the relation between thermal E.M F and the temperature difference between inside and outside $y = 11\ 35\ x$ where y in mV x in C

b) the relation between temperature and heat input $y = 0\ 042\ x + 1\ 2$ where y in C x in Watts The constant represents the heat by the illumination

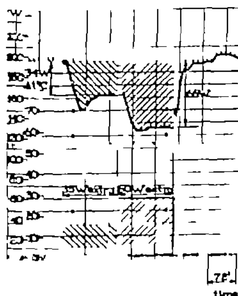


Fig 2 11

Record of a dynamic calibration of the gradient calorimeter by applying known amounts of electrical energy When the permanent heat input is changed, the variable heat input show the same change with reversed sign. These two measurements are also seen in Fig 2 1

c) the relation between heat input and the deflection of the recorder $y = 2.56\ x - 1.5$ where y in Watts, x in scale units.

d) *the relation between* thermal gradient and deflection of the recorder $y = 0.12\ x - 0.12$ where y in °C x in scale units.

e) *detection of a known extra heat input.* A record of such an experiment is given in Fig. 2.11 By changing the permanent heat input, known from an accurate Watt meter from one level to another an increase or decrease of the variable heat input could be obtained Deflections to both sides were investigated and are presented in Fig 2.12 A positive sign means a higher permanent

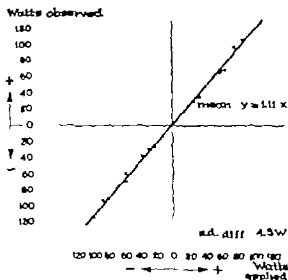


Fig 2.12.

Dynamic calibration of the gradient calorimeter A positive sign means a higher permanent heat input (imitating heat dissipation by the subject), negative sign a lower one (imitating heat absorption by the subject)

heat input (imitating heat dissipation of the subject) a negative sign a lower one (imitating heat absorption by the subject) There is a systematic error of 11 % for which no explanation can be given and a standard deviation of 4.5 Watts for the differences from the mean line This equals 0.06 kcal/min. The gradient calorimeter has at least two time constants 3.4 and 18.2 min.

2 5 Ambient conditions

In this study the subject was exposed successively to two different environmental conditions one while sitting in the gradient calorimeter and the other in the climatic room while walking on the treadmill This climatic room is in fact only an air temperature controlled room The accuracy is 0 5 C the reproducibility still worse

The following climatic factors are not measured during the experiments but have been investigated previously

a) Wall temperatures. From preliminary investigations using Vernon s globe thermometer it was thought to be safe in assuming them equal to air temperatures under the actual experimental conditions.

b) Radiant heat sources such as illumination in both rooms and the heating elements in the gradient calorimeter were not considered separately since the spatial angles towards the subject were such that they had only little effect

c) Wind velocity In preliminary measurements the wind velocity was determined with a home made hot junction anemometer which was calibrated while mounted on a 2 5 m long wing rotating around with a known constant velocity in a completely closed room In the gradient calorimeter a mean value of 125 cm/sec was observed In the climatic room a mean velocity of 8 cm/sec was measured one meter above the belt of the treadmill. In the severest experiments (air temperature 35 C net heat production 7½ kcal/min) the air velocity was increased to a mean value of 83 cm/sec with a blower aspiring room air and directing it towards the subject

The following ambient factors were measured during the experiments

d) Air temperatures were measured with shielded thermometers once a cycle (7 2 min) One was mounted behind the observation window of the gradient calorimeter and one on the wind generator in front of the treadmill.

e) Humidity is not controlled in the present climatic rooms It is measured with a lithium-chloride dew point meter together with the air temperatures Since the experiments were carried out during winter time the requirement to keep the water vapour

pressure low in order to facilitate the evaporation of sweat was met without effort.

f) Treadmill speed and inclination. The treadmill consisted of a motor-driven rubber belt with adjustable speed up to 10 m/sec, mounted on a frame which could be tilted around an axis located in the middle between the two end-rollers. The rear roller was driven with V belts by a pulley the axis of which was in line with the axis of the frame. This set-up made it possible to go uphill or downhill with the belt always moving in the same direction. The maximum inclinations were 15 degrees. A control system counteracted the slight increase in speed with time which was probably due to the change in temperature of the oil in the hydraulic variator. The fluctuations in speed were less than 1 cm/sec in the speed ranges used. The inclination was read on a round dial, the indicator of which makes one complete revolution for 15 degrees inclination and the scale divisions were 0.0* degrees. Speed and inclination were measured with each determination of the metabolism. The desired walking speeds and inclinations were calculated once for each subject with BOBBERT's equation (1960) and maintained throughout.

.6. METABOLISM

The metabolic rate was measured with Douglas bags and duplicate Haldane analysis. The inspired air was room air which had standard composition due to sufficient fresh air supply to both climatic rooms. The volumes of the Douglas bags were measured with a calibrated chain-compensated 350 liter Tissot spirometer. The efficiency of the Haldane procedure was increased by using three apparatus in a row and moving the mercury reservoirs with motor driven vertical chains (MUELLER 1930). For all three apparatus the standard deviations of the differences for CO_2 and O_2 were 0.0* and 0.03 respectively. *7 consecutive duplicate analyses of one sample on two different apparatus give 0.03 and 0.05 respectively. V_{STPD}, RQ and V_{O_2} were calculated in the usual way (CARPENTER, 1930). The metabolic rate in kcal/min was calculated with a slightly modified equation according to WEIR (1949). From WEIR's deductions the following equation may be obtained for the metabolic rate

$$\dot{M} = \frac{3.941 - 1.106\,R}{(1 + 0.082\,p)\,\{1 - F_{I_{O_2}}\,(1 - R)\}}\;\dot{V}_E\,(F_{I_{O_2}} - F_{E_{O_2}})$$

where $\dot{M}$ = metabolic rate in kcal/min

p = fraction of the total heat production due to protein combustion

R = respiratory quotient

$\dot{V}_E$ = expiratory minute volume STPD

$F_{I_{O_2}}$ = Oxygen fraction in inspired air

$F_{E_{O_2}}$ = Oxygen fraction in expired air

The first term on the right side is nearly a constant. WEIR introduces a value of 12.5 % for p but this value is certainly too high during exercise since it is known that protein combustion does not increase during exercise (ZUNTZ und SCHUMBURG 1901). By calculating the first term for all combinations of R = 1.0, 0.95, 0.90, 0.85, 0.80 and 0.75 and p = 12.5 %, 10.0 %, 7.5 %, 5.0 %, 2.5 % and 0.5 % an average of 5.015 is obtained with a coefficient of variance of 3.0 %. The equation used is therefore

$$\dot{M} = \dot{V}_E\,(1.05 - 5.015\,F_{E_{O_2}})$$

Analysis of the duplicate determinations during sitting in the gradient calorimeter and of the quadruple determinations during walking revealed a standard deviation of 0.05 and 0.10 respectively. When the subject walked uphill the net metabolic rate was calculated by subtracting the caloric equivalent of the external work from the gross metabolic rate (SNELLEN 1960).

2.7 WEIGHT LOSS AND SWEATING RATE

In this study the sweating rate was obtained from weight loss. Since the sweating rate is very important in this study every precaution was taken to secure that all produced sweat be evaporated as fast as possible:

1) the subjects were clad to a minimum. Their clothes were never soaked with sweat even at high sweating rates (> 10 g/min)

2) the water vapour pressure was low (average 4.7 mmHg)

3) if necessary the wind velocity was increased during work

when it did not interfere with the heat exchange by convection. In the gradient calorimeter the wind velocity was always high

Both continuous recording of the weight loss and intermettent weighing were applied.

2.7 1 *Continuous recording of the weight loss when the subject was sitting in the gradient calorimeter*

The gradient calorimeter was mounted on a specially constructed 1 2 balance as shown in Fig 2.8 The weight of the gradient calorimeter plus the subject was counterbalanced to within 3 kg the remaining 2 kg being indicated on a dial. On the axis of the indicator a low torque potentiometer was mounted which was part of a Wheatstone bridge. The recorder and the Wheatstone bridge were so adapted to each other that full scale deflection (21 cm) was obtained for approx 200 g After critical electrical damping of the recorder the balance was critically damped with a plunger in oil. The connecting wires to and from the gradient calorimeter were made of flexible material and as thin as the requirements permitted. They hung freely in the air in wide loops. The same holds for the corrugated rubber expiration tube. The maximal displacement of the plateau was 32 mm but in the actual experiment it was not more than 2.4 mm. Two records are shown in Fig 2.12 The accuracy of static absolute weighings was within 5 g The accuracy with which weight losses per minute could be measured was determined by analysing the observations during steady state conditions. In this study the weight loss per minute was determined twice a cycle (7.2 min) by differentiating the obtained curve over 3 6 min. 13 series of 5 observations, made at the end of one hour s sitting in the gradient calorimeter including three different levels of weight losses per minute (mean values 0.5 2.4 and 4.2 g/min) revealed a standard deviation of the differences of 0 14 g/min which equals approx. 0.00 kcal/min.

7.— *I termittent weighing*

During the period of work and heat load the subject s body weight was measured four times at 1 6 minutes intervals (3 cycles) on a 1 1 balance accurate to within 2 grams for non-moving

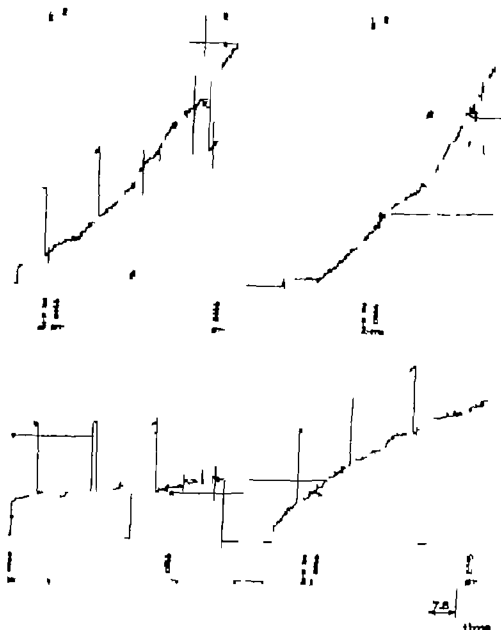


Fig 13

Two specimens of continuous records of weight loss while the subject sits in the gradient calorimeter Time base 3 6 min intervals. Checks of electrical zero and repeated calibrations with 50 g Left hand part during the first period of sitting in the gradient calorimeter right hand part during the recovery period

Upper record: subject v T experiment 16 Note the lag of approx 8 min before sweating starts again.

Lower record subject W.K., experiment 27

objects In addition, the weight was determined immediately before and after the whole experiment. Thus six body weight determinations were obtained, denoted as G_0, G_1 G_2 G_3, G_4 and G_5. For the accuracy of weighing subjects see 3.2.2..

7.3 *Corrections*

The weight loss due to exchange of respiratory gases was calculated from the equation

$$\Delta G_{ge} = \dot{V}_{O_2}\ (1\ 9769\ R - 1\ 42904)$$

where ΔG_{ge} = weight loss in g/min due to gas exchange
$\dot{V}_{O_2}$ = oxygen consumption in liters/min STPD
R = respiratory quotient
1 9769 = weight in grams of one liter CO_2 STPD
1 42904 = weight in grams of one liter O_2 STPD

The weight loss due to evaporation of water in the respiratory tract was calculated from the equation

$$\Delta G_{rt} = f_1\ \dot{V}_E\ a - f_2\ \dot{V}_I\ b$$

where ΔG_{rt} = weight loss in g/min due to evaporation in the respiratory tract
$\dot{V}_E$ = expiratory minute volume STPD
$\dot{V}_I$ = inspiratory minute volume STPD $\left(\dot{V}_I = \dot{V}_E\ \frac{F_{E_{N_2}}}{F_{I_{N_2}}}\right)$
a = water vapour density of the expired air in g/liter dry air at expired air temperature
b = water vapour density of the inspired air in g/liter dry air at dew point temperature
f_1 f_2 = conversion factors for STPD to BTPS and ATP respectively

Since the expired air is always fully saturated with water vapour factor a is approx. 34 mg/liter (HOUDAS et COLIN 1966)

The heat of vaporisation of one gram sweat has been calculated with the equation given by HARDY (1949)

$$H = L + T_{ew}\ (\varphi_2 - \varphi_1) + 0\ 1104\ T_s\ \ln\ (RH)\ 1$$

where H_v = heat of vaporisation in cal/g

L = latent heat of vaporisation

T_{ave} = average absolute temperature $\frac{1}{2}(T_s+T_0)$

φ_2 = entropy of the liquid + vapor at room temperature

φ_1 = entropy of the liquid + vapor at skin temperature

T_0 = absolute room temperature

RH = relative humidity

Strictly speaking the heat of vaporisation is different in the respiratory tract and on the skin. This difference was neglected.

The heat loss by evaporation was calculated from the heat of vaporisation and the weight loss corrected for the weight loss due to gas exchange called ΔG Sweating rate was called $\Delta G'$ indicating that two corrections were made

2 8 Statistics

The statistical techniques applied in this study were all derived from a Dutch textbook by H de Jonge (1963)

Fig 2.14

Synoptic table of one experiment.

2.9 Sequence of events in one experiment

Previous day 1) both climatic rooms set at desired temperature
2) treadmill speed and inclination set
3) gradient calorimeter set at desired gradient
4) breathing valves mounted.

Experiment day 1) 24 point recorder set in operation. This recorder serves as time base Each cycle takes 7.2 min.
2) subject reports to the laboratory at 08.30
3) Douglas bags rinsed and emptied
4) subject is dressed in the "harness"
5) the actual experiment is shown in the synoptic table (Fig 2.14).
6) 14.00–16 00 Haldane gas analyses.

3. EXPERIMENTAL RESULTS

3 1 Presentation of results

Only few of the primary data can be used directly (e.g air and dew point temperatures) Others serve for the computation of numerous derived magnitudes (e.g metabolism, weight losses, non-evaporative heat exchange body temperatures etc.) These calculations have been described in Chapter 2. Both groups of data represent the basic material which is, even for one subject much too large to be presented as a whole in this paper but several groups of basic data can be combined after further arithmetical treatment, which will be discussed below These combinations serve either as evidence for a statement or as cross checks on the collected data. The experimental results will be presented in three categories steady state conditions, changes with time and functional relations.

3.2. Steady state conditions

3.2.1 *At the end of the first period of sitting in the gradient calorimeter*

3.2 1 1 *Reproducibility* During the first hour of sitting in the gradient calorimeter the subject should reach a steady state before he starts his period of work and heat load. When the results of different combinations of work and heat load starting from one particular gradient-calorimeter temperature are to be compared it is desirable to have the subject in a condition as nearly identical as possible prior to these various working periods. Attempts to reach

this goal were reasonably successful Table 3 1 shows the averages and standard deviations of body temperatures together with those of other physical and physiological variables of the subjects.

TABLE 3 1

Means and standard deviations of physical and physiological variables observed at the end of the first period of sitting in the gradient calorimeter

Grad Calor	Subject		t	t	t	M	$P+O$ kcal/min	ΔG/min	G_1	10_{b-1}
	W K	m	30 0	36 70	3* 06	1.30	−1 01	0.57	79 700	57
30		s.d.	1 0	0 15	0 4	0 09	0..3	0..7	0 723	14
	V T	m	30 5	36.65	33.20	1 60	−0 95	1 06	74 613	64
		s.d	1 0	0 1*	0 48	0 06	0 18	0.36	0.509	16
	W K	m	35 *	37 03	34 6	1 38	+0 19	,35	76.849	147
35		s d	0 6	0 18	0..3	0 07	0 10	0 30	0..65	23
	V T	m	34 0	36.7	34 07	1.58	+0 04	2.57	74 018	133
		s.d.	0 6	0 08	0..0	0 15	0 09	0 35	0.168	15
	W K	m	40 0	36 98	36.10	1 43	+1 43	4 15	79 384	243
40		s.d	1.8	0 05	0 4	0 06	0 50	0 65	0 650	*3
	V T	m	40 8	36 78	36 55	1 63	+1 44	4 9*	74.313	*62
		s.d	1 0	0 06	0 43	0 06	0.57	0 73	0 643	65

3 2 1 2 *Non-evaporative heat exchange* The non-evaporative heat exchange plotted against the temperature difference between air and weighted mean temperature of the thermojunctions of the harness gives the heat exchange per degree difference (Fig 3 1) for the subjects Provided that the slope of the lines in Fig 3 1 i e the heat exchange per degree difference is in good agreement with data from the literature the individually different distance from zero on the abscissa may be regarded as an expression of the error of the harness for each subject (see 2 3 2 3)

The Stefan Boltzmann equation (see HARDY 1949) may be rearranged to a linear form assuming the emissivities to be unity and $(T^4 - T^4)/(t - t)$ to be a constant. The latter assumption is safe to within 3 % when $(t - t)$ does not exceed 0 C:

$$R = 5\,8\ f_R\ A\ (t - t)$$

where R = heat exchange by radiation in kcal/h

f_R = ratio of the effective radiating surface area to the total body surface area

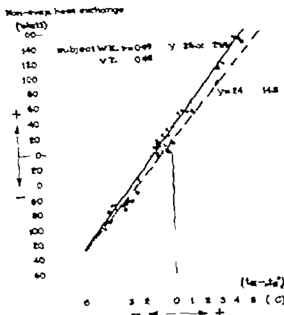


Fig. 3 1

Non-evaporative heat exchange in Watts of both subjects, plotted against the difference between air temperature (t_a) and mean temperature of the thermojunctions ("t_s").

A = body surface area in m²
t_w = mean wall temperature in °C
t_s = mean skin temperature in °C.

From the conclusions drawn by [illegible] *et al.* (1938) and CLIFFORD *et al.* (19[illegible]9) the following equation for the heat exchange by convection may be derived

$$C = 0.243\ v^{[\text{illegible}]}\ f\ A\ (t_s - t_a)$$

where C = heat exchange by convection in kcal/h
v = wind velocity in cm/sec
f = ratio of effective convecting body surface area to the total body surface area
A = body surface area in m²
t_a = air temperature in °C
t_s = mean skin temperature in °C.

Assuming $f_R = 0.75$, $f_C = 0.07$ ([illegible] 1930) and $t_w = t_a$, neglecting heat exchange by conduction, introducing the observed data for A and v (W.K. 1.96 m², V.T. 1.90 m², v = 125 cm/sec) and

converting kcal/h into Watts the following constants are obtained by adding both equations 26 W/°C for W.K and 25 W/°C for v T The agreement is so close that it seems justified to conclude that the error of the harness is 1 15 C for W K and 0 00 C for the leaner v T

3 2 1 3 *True mean skin temperature as a function of the air temperature* It may be seen from Fig 3 2 that the relation between these two temperatures is nearly a straight line and that the leaner v T has a slightly higher skin temperature than W K

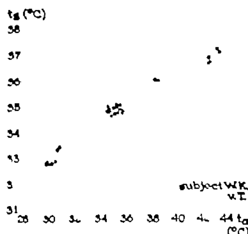


Fig 3 *

Air temperature and true mean skin temperature observed at the end of the first period of sitting in the gradient calorimeter

3 2 1 4 *Heat balance equation* If at the end of one hour s exposure the sum of heat production and the non-evaporative heat exchange both per minute ($\dot{E}_{req}$ after BELDING and HATCH 1955) equals the product ($\dot{E}$) of the corrected weight loss per minute ($\Delta G'$/min) and the heat of vaporisation it may be assumed that a steady state has been achieved for this situation In Fig 3 3 it is seen that most results fall well within a band of plus or minus 0 6 kcal/min i e two times the standard deviation of the differences. This is the accuracy of the method Since there is no appreciable deviation from the diagonal or line of identity (mean difference for W K +0 07 for v T − 0 03 standard deviations 0 284 and 0 300 respectively Student s *t* test for differences with double-

tailed probability more than 0 05 and 0 50 respectively) the above-mentioned assumption is safe within the accuracy of the measurements.

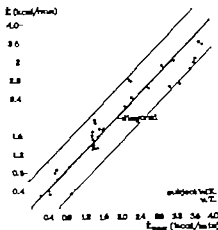


Fig. 3.3.

Heat balance at the end of the first period of sitting in the gradient calorimeter

3 2. *At the end of the period of work and heat load*

3.2.1 *Final rectal temperature as a function of metabolic rate.* It is generally accepted that rectal temperature during work is largely a function of the metabolic rate and independent of ambient conditions over a wide range (NIELSEN and NIELSEN 1962) In this study one subject, W.K. showed, however the striking phenomenon that his final rectal temperature was the higher at the same metabolic rate the higher the temperature had been in the preceding period of sitting in the gradient calorimeter (Fig 3 4) The three regression lines (solid lines) have different regression coefficients, but their differences are not significant (analysis of covariance) The temperatures at the points where the three regression lines with one common regression coefficient (dotted lines) cut the ordinate (a-coefficients) differ however highly significantly This is not due to an elevated starting rectal temperature (Table 3 1) It is more likely to be due to the preceding dehydration. This relation between the a-coefficients and preceding dehydration

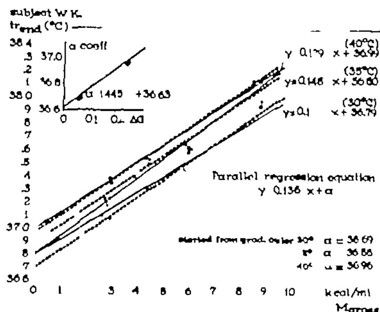


Fig 3 4
Rectal temperature of subject W K at the end of the period of work and heat load as a function of gross metabolic rate. For analysis of covariance see text

is shown in the inserted graph The other subject does not show any significant difference in the α-coefficients as shown in Fig 3 5 He obeys the classical concept that the final rectal temperature is a function of the metabolic rate only

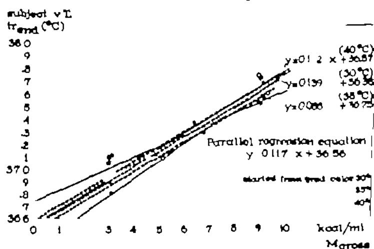


Fig 3 5
Rectal temperature of subject v T at the end of the period of work and heat load

The reason why rectal temperature is plotted against gross metabolic rate instead of net metabolic rat is based on the finding that rectal temperature seems to be independent of mechanical efficiency as shown in Fig 3.6.

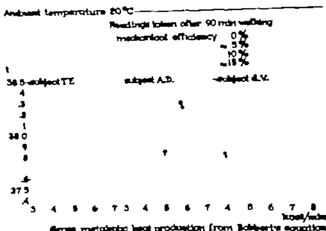


Fig. 3.6.

Results f an attempt t detect any effect of external work on rectal temperature Metabolic rat was not measured but estimated using BOBBERT' equation.

The subjects walked on the treadmill t different speeds and inclinations providing six different levels of metabolic rat per kg body weight with slopes of 0, 0.05 0.10 and 0.15 for the mechanical efficiency or $U/(G\ E\ )$. U and E are expressed in the following equations

$$U = 0.02342\ G \quad \sin$$

$$\log E = 1.4272 + 0.004591 \quad + 0.024487\ \alpha + 0.0002838$$

(BOBBERT 1960)

where U = external work per minut in kcal/min

G = body weight in kg

= walking speed in m/min

= inclination in degrees

E_w = metabolic rat in cal/kg min.

Sin may be regarded to be directly proportional t α between 0 and 6 degrees. The highest inclination required was 5.05 degrees. Although the metabolic rat was not measured, Bobbert s equation for its computation, as presented above seems accurat enough in healthy male subjects (within 10) according t the author experience, to warrant this conclusion.

3 2 2 2 *Weight losses* Three measurements are made during the period of work and heat load denoted as ΔG_2 ΔG_3 and ΔG_4 The second and the third measurements are compared in Fig 3 7 in order to ascertain that during the exposure to work and heat load the subject has reached a constant sweating rate The data scatter at random around the diagonal or line of identity (nearly equal

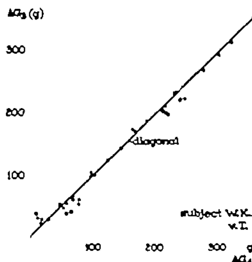


Fig 3.7

Comparison of the second (ΔG_3) and the third (ΔG_4) measurement of weight loss during the period of work and heat load.

numbers of points above and below the line a rank correlation of 0 06 between the differences of the pairs and their means) The mean difference is -0 625 and the standard error of this mean is 2 85 The standard deviation of a single average ($\frac{1}{2}\Delta G_3 + \frac{1}{2}\Delta G_4$) estimated from these differences is 8 84 This equals 0 41 g/min It is therefore admissible to use the average of both determinations. The weight losses due to evaporation $\Delta G'$/min and the sweating rates *(S.R.)* ΔG /min during the period of work and heat load are calculated in the subsequent sections from these averages

3 2 2 3 *Heat balance equation* During the period of work and heat load there is no continuous measurement of the non-evaporative heat exchange Nor can it be assumed that the error of the harness in a moving subject in a completely different air velocity is the same as while sitting in the gradient calorimeter Nevertheless it

is possible to investigate the heat balance the observed net metabolic heat production (M_{net}) the weight loss due to evaporation ($\Delta G'$/min) and the difference between air temperature and mean temperature of the skin thermojunctions ($t_a - t_s''$) are plotted in one graph with the assumption that a steady state has been reached. Since the metabolic rate shows the best reproducibility it is used

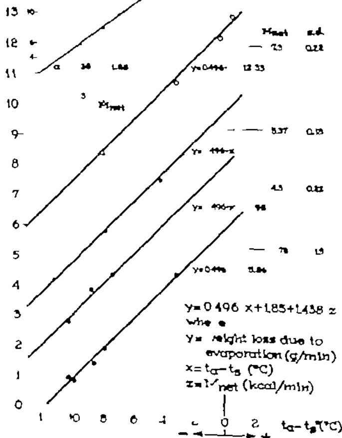


Fig. 3.8.

Subject W.K. Weight loss due to evaporation ($\Delta G'$) plotted against the difference between air temperature (t_a) and mean temperature of the thermojunctions ("t_s"), at four levels of metabolic heat production. For the analysis of covariance see text.

as a parameter in the plot of the weight loss against the temperature difference The results are shown in Figs 3 8 and 3 9 Four straight lines are obtained per subject with regression coefficients which do not differ significantly (analysis of covariance) The common regression coefficient is used to draw the lines shown This coefficient

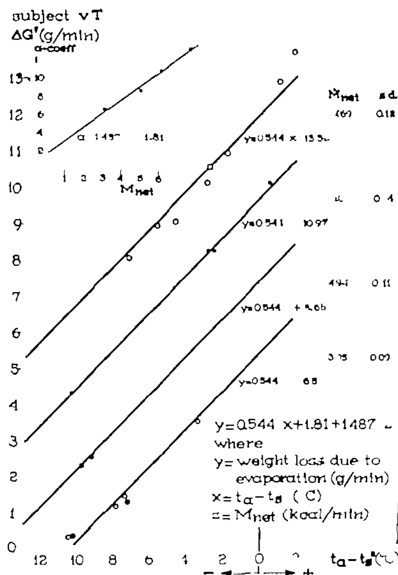


Fig 3 9

Subject v T Weight loss due to evaporation ($\Delta G'$) plotted against the difference between air temperature (t_a) and mean temperature of the thermojunctions (t_s) at four levels of metabolic heat production. For the analysis of covariance see text.

has the dimension of (g/mm)/degree The values of $\Delta G'$/min at $(t - "t")=0$ (a-coefficients) plotted against the metabolic rates (inserted graph) gives a straight line with a regression coefficient with the dimension of (g/min)/(kcal/min) By dividing the first coefficient by the second the non-evaporative heat exchange per degree temperature difference is obtained (kcal/min)/degree. The reciprocal of the second coefficient gives the heat of vaporisation (kcal/g) Finally the y-intercept in the inserted graph gives the weight loss at $M=0$ and $(t - t_s)=0$ or when divided by the first coefficient, the error of the "harness" in degrees Celsius. The results are shown in Table 3.2.

TABLE 3.2

Coefficients for non-evaporative heat exchange heat of vaporisation, and error of the harness obtained from analysis of Figs 3.8 and 3.9

Subject	(kcal/min)/degree	kcal/g	error "harness
W K	0.345	0.693	3.71 C
T	0.366	0.672	3.33° C

The heat of vaporisation is slightly higher than the average value of 0 65 as calculated with Hardy's equation (HARDY 1949) This difference cannot be explained. Either one or more of the data have a systematic error due to a possible slight deviation from

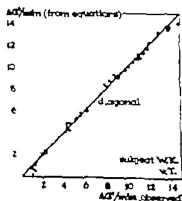


Fig 3 10

Comparison of weight losses due to evaporation as actually observed and as calculated from the equations presented in Figs. 3.8 and 3.9

the steady state or Hardy's equation underestimates the heat of vaporisation during walking If the former is the case then the above mentioned assumption of a negligible rate of heat accumulation is incorrect By inserting all observed data in the obtained equations however the actual weight loss may be compared with that calculated (Fig 3 10) The agreement is very close and there is not the slightest indication that lower values where the rate of heat accumulation may certainly be neglected are different from those where this is questionable Therefore the assumption of the presence of a steady state seems to be permitted

3 2 2 4 *The true mean skin temperature as a function of the air temperature.* It may be seen from Fig 3 11 that the relation is

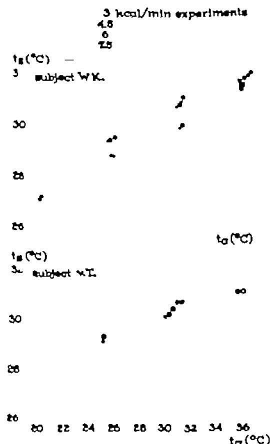


Fig 3 11
Air temperature and true mean skin temperature observed at the end of the period of work and heat load

nearly a straight line No distinct influence of the work load can be detected. The effect of locomotion as such, however can be seen by comparing Figs. 3 11 and 3._.

3.2.3 *At the end of the recovery period*

3.2.3 1 *Heat balance equation.* All physiological responses and variables more or less return to their original value observed at the end of the first period of sitting, with the exception of the rectal temperature and the body weight. Metabolism is usually slightly lower (not shown) as is the mean skin temperature (Fig 3 15). Sweating rate may not return to its original value either not only because the subject became so warm during the period of work and heat load that at the end of the recovery period he still sweats in order to dissipate this excess heat, but also because metabolism and/or non-evaporative heat exchange are altered. The same technique as applied for Fig. 3 3 is used to investigate whether the heat balance again reached a steady state within the "accuracy of the measurements at the end of the recovery period (Fig 3 1).

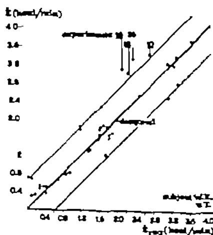


Fig. 3.12.

Heat balance at the end of the recovery period.

Four experiments fall clearly outside the band of 0 6 kcal/min but they do not concern the severest loads (see Fig. 2_ and 2.3) as might be expected. The statistical analysis, however reveals,

even after omitting these four observations that $\dot{E}_{req}$ and $\dot{E}$ are not identical (mean difference for W K −0 106 for v T −0.139 standard deviations 0 350 and 0 236 respectively Student's t test for differences with double tailed probability smaller than 0 0* and 0 05 respectively) With the exception of the four observations it seems admissible to state that heat balance has been practically regained It may be unfortunate that the length of the recovery period could not be extended beyond one hour

3 2 3 2 *Elevation of rectal temperature as a function of preceding dehydration* The rectal temperature remains elevated as compared with its starting level in proportion to the weight loss in the intermediate period This is shown in Figs 3 13 and 3 14 Those experi

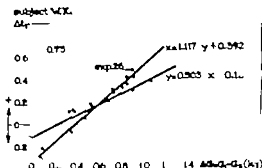


Fig 3 13

Subject W K The differences in rectal temperature observed at the end of the first period of sitting in the gradient calorimeter and the end of the recovery period plotted against the losses of body weight in the same time interval

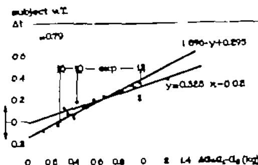


Fig 3 14

Subject v T The differences in rectal temperature observed at the end of the first period of sitting in the gradient calorimeter and the end of the recovery period plotted against the losses in body weight in the same time interval

ments which deviated distinctly in Fig 3 12 are indicated. The line of subject W.K. who showed an influence of preceding dehydration on his final rectal temperature during the period of work and heat load (Fig 3 4) has a steeper inclination than that of the other subject. The rectal temperature of subject v T seems to be less affected by dehydration. This may account for his failure to demonstrate any effect during the period of work and heat load The objection might be raised that rectal temperature has not yet returned to its final level since heat balance has not been entirely reestablished at the end of the recovery period (3 2.3 1) The probable effect of dehydration, however is further confirmed by another observation (see 3 4.2.) yielding almost the same regression coefficients.

3.3 Changes with time

Body temperatures were recorded in short (3 6 min) intervals throughout each experiment. During the sitting periods body weight and non-evaporative heat exchange were recorded continuously It has not been, however the primary aim of this study thoroughly to investigate the transient conditions of changing from sitting to walking and vice versa. It would have been extremely interesting to make continuous recordings of the decline of the sweating rate as quickly as possible after cessation of work, but usually it took the subject several minutes to enter the gradient calorimeter connect his "harness" to the recorders insert the mouthpiece of the breathing valve and sit quietly again to set the balance into operation. Therefore no recordings could be made during this essential period The same applies to the non-evaporative heat exchange The necessity of readjusting the previous conditions in the gradient calorimeter by replacing the heat lost during opening of the door with the extra heating element caused a disturbance lasting several minutes and interfering with accurate continuous recordings. Finally not all measuring devices were investigated thoroughly for their time constants which might interfere with the time constants of the physiological variables under study Therefore this aspect of the results will be reviewed only briefly and only for subject W.K. since in his case the experiments were carried out completely according to schedule (Fig 3).

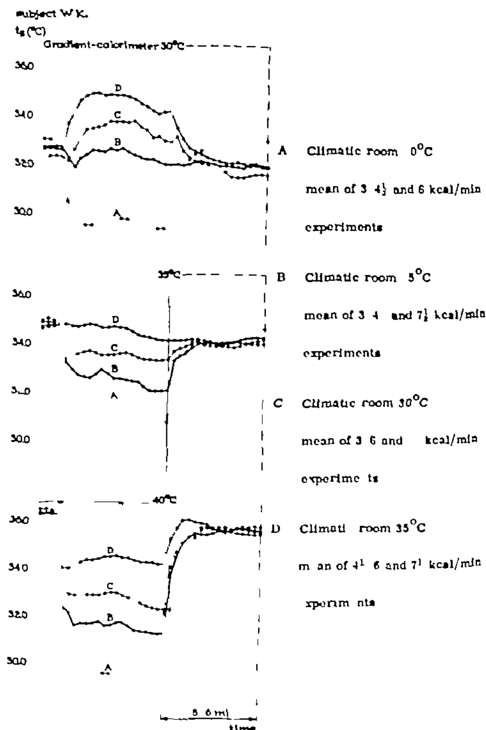


Fig 3 15.

Changes in mean skin temperature with time. Averages of comparable experiments of subject W.K

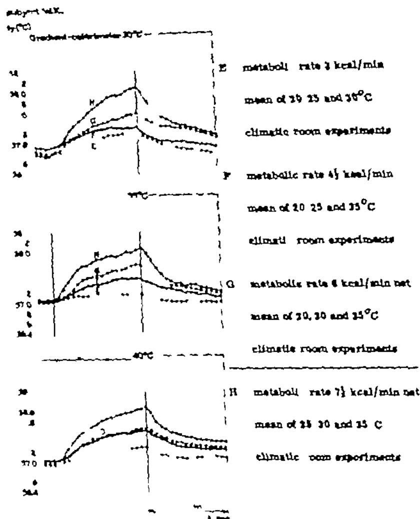


Fig. 3.16.

Changes in rectal temperature with time. Averages of comparable experiments of subject W.K.

3.3.1 *Body temperatures*

According to Figs. 3.2 and 3.11 the mean skin temperature in steady state conditions is only influenced by the ambient temperature and not affected by metabolic rate. Assuming that this is

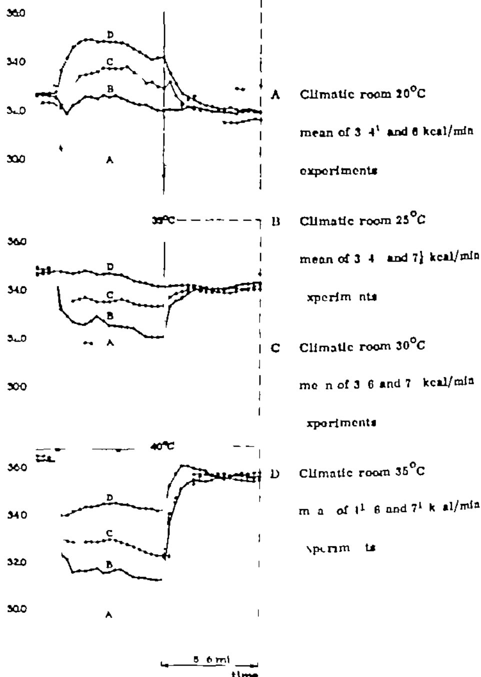


Fig 3.15.

Changes in mean skin temperature with time. Averages of comparable experiments of subject W K.

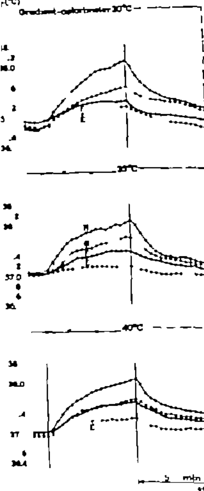


Fig 3.16.

Changes in rectal temperature with time. Averages f comparable experiments of subject W.K.

3.3.1 *Body temperatures*

According to Figs. 3.2 and 3 11 the mean skin temperature in steady state conditions is only influenced by the ambient temperature and not affected by metabolic rate. Assuming that this is

also the case in transient conditions the mean skin temperatures in all experiments carried out in the same ambient conditions have been averaged and plotted against time (Fig 3 15) In subject W K there is a statistically significant influence of the conditions before the period of work and heat load on the final rectal temperature during this period (Fig 3 4) Apart from this the rectal temperature is only influenced by the metabolic rate Assuming that this also holds in transient conditions the rectal temperature of all experiments with the same metabolic rate and initial conditions have been averaged and plotted against time (Fig 3 16) The experiments of subject v T do not reveal any essential difference but taking the averages of his data is impaired because the experiments on v T were not carried out according to schedule

3 3 2 *Changes in sweating rate*

Only the transition of work to rest has been investigated with continuously recording techniques with the limitations mentioned above It was not possible to average all readings in any way without loss of information Therefore not all recordings of subject W K will be presented but only those with prominent features to illustrate the findings.

Two distinct types of decline in sweating rate were observed.

3 3 2 1

A slow decline is seen under those conditions where ambient temperatures during walking and sitting were equal and close to the mean skin temperature Under these conditions the subject does not have any appreciable possibility to dissipate heat by radiation and convection

Four experiments are available with four metabolic rates during walking They are shown in Fig 3 17 Whereas two curves show a smooth decline two others show a fluctuation in sweating rate An explanation is hazardous since technical imperfections cannot be excluded An analysis of experiment 2 (inserted graph) reveals a time constant ($1/e$) of roughly ten minutes There are three experiments available where metabolic rate is low and where the

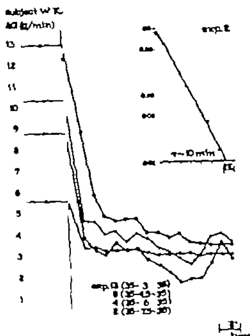


Fig. 3.17
The fall in sweating rate after exercise. Four observations on subject W.K. where non-evaporative heat exchange is small.

sweating rate is low due to a combination of this low metabolic rate with a low ambient temperature. When changing from low to high sweating rates, e.g. as required at a high gradient-calorimeter temperature the rate of change in sweating rate is slow too (Fig. 3.18) Analysis of experiment 28 (inserted graph) reveals a time constant of roughly 13 minutes.

3.3.2.2.

The fast component was not actually observed but its existence was deduced from the slow component. Three experiments with large changes in metabolic rates and with high sweating rates returning to low levels are available (Fig. 3.19) Here again not all curves are smooth. A semilogarithmic plot of the data of experiment 27 shows a more or less straight line for the actually observed

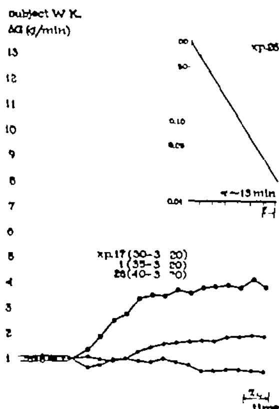


Fig 3.18.

Changes in sweating rate after exercise. Three observations on subject W K where sweating rate during exercise was low

data (inserted graph) with an estimated time constant of about 14 min which is comparable to that of experiments 28 and 2 mentioned above but extrapolation from the first measured value *to the starting point reveals an initial steeper line indicating* a fast decline at the beginning The time constant of this initial fast component may be estimated to be of the order of a few minutes and is in the same order of magnitude as the rate of change of the metabolism after cessation of moderate work (HENRY and DE MOOR, 1955)

3 3 3 *Accumulation of heat in the body*

The heat balance equation may be applied also over periods of time in order to evaluate the heat accumulated in those periods

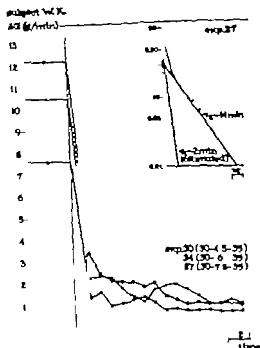


Fig. 3.10

The fall in sweating rate after exercise. Three observations on subject W.K. where high sweating rates return to low values.

when it has been demonstrated that at the beginning and at the end of this period the rate of heat accumulation is negligible. This has been shown to be the case for the initial sitting period, for the period of work and heat load and nearly so for the period of recovery (Figs. 3.3, 3.8 and 3.9–3.12)

3.2.3.1 Accumulation of heat in the body in the period of work and heat load. In Table 3.2 the coefficients for non-evaporative heat exchange and the heat of vaporisation were presented. These factors may be applied to the sum of the differences between air and mean skin temperatures and to the total corrected weight loss during the exercise period, respectively

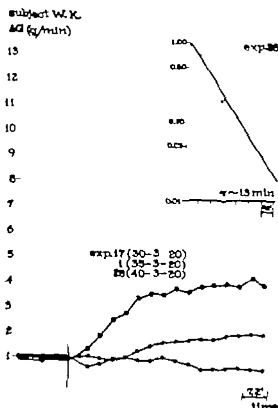


Fig 3 18.

Changes in sweating rate after exercise. Three observations on subject W.K. where sweating rate during exercise was low

data (inserted graph) with an estimated time constant of about 14 min which is comparable to that of experiments 28 and 2 mentioned above but extrapolation from the first measured value to the starting point reveals an initial steeper line indicating a fast decline at the beginning The time constant of this initial fast component may be estimated to be of the order of a few minutes and is in the same order of magnitude as the rate of change of the metabolism after cessation of moderate work (HENRY and DE MOOR, 1955)

3 3 3 *Accumulation of heat in the body*

The heat balance equation may be applied also over periods of time in order to evaluate the heat accumulated in those periods,

There are quite a few objections to be made against this procedure but this hardly deserves attention because the heat involved is of minor importance numerically.

$(R+C)_{rec}$ was calculated as follows. If the number of Watts required to maintain the thermal gradient of the calorimeter without subject is represented by A (average of 12 readings) and the sum of the number of Watts observed in the whole recovery period (16 readings) by ΣW, then the total non-evaporative heat exchange is

$$(R+C)_{rec} = 2.6\ (16A - \Sigma W)\ 0.89\ 0.014327$$

where $(R+C)_{rec}$ in kcal

- 2.6 time interval in minutes between two readings
- 0.89 11 % correction (see 2.4)
- 0.014327 conversion factor (kcal/W)

3.2.3.3 *Comparison of the heat accumulated in the period of work and heat load and lost in the recovery period.* It is obvious that the calculation of ΔH_1 is much less accurate than that of ΔH_2. While in the latter all the left hand terms were measured more or less continuously, in the former the observed working metabolism was multiplied by a large number of minutes, neglecting the gradual rise in metabolism at the start, and the non-evaporative heat exchange was calculated rather than measured. With these restrictions the two determinations show a fair agreement, as shown

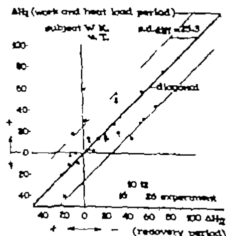


Fig. 3.20.

Comparison of the heat accumulated in the body during the period of work and heat load and the heat lost in excess in the recovery period.

Hence

$$\Delta H_1 = M_{1tot} + \Delta G_w \ H_{v_1} + (R+C)_{tot}$$

where M_{1tot} = M_{net} multiplied by 64 8 (minutes)

ΔG_w = corrected total weight loss during the period of work and heat load $\Delta G'_3 + \Delta G_3 + \Delta G'_4$

H_{v_1} = heat of vaporisation as calculated (Table 3 9)

$(R+C)_{tot}$ = 3 6 $\Sigma (t_a - t)$ coefficient (Table 3 2)

3 3 3 2 *Loss of the accumulated heat in the recovery period* In the recovery period the heat production by metabolism the total weight loss due to evaporation and a continuous record of the non evaporative heat exchange are available The heat lost in excess to the heat produced and/or gained in this period is

$$\Delta H_2 = M_{2tot} + \Delta G_5 \ H_{v_2} + (R+C)_{2tot}$$

where M_{2tot} = the total heat produced during the recovery period

ΔG_5 = corrected total weight loss during the recovery period

H_{v_2} = heat of vaporisation from Hardy s equation

$(R+C)_{2tot}$ = total non-evaporative heat exchange in the recovery period

M_{2tot} was calculated from the four determinations in the recovery period, by multiplying the observed values by the filling times of the Douglas bags and adding these figures. The time elapsed between the 2nd and 3rd determination was multiplied by the average of these two. The heat production in the remaining period between end of exercise and start of the first determination was estimated with the following assumptions and simplifications:

1) the oxygen debt consists only of the alactacid component (MARGARIA et al 1933) The time constant for this component is 1.3 min (HENRY and DE MOOR, 1955) The time to reach the metabolic rate of the first determination can then be calculated

2) the decline in metabolic rate in the above mentioned period may be regarded as linear or the heat produced in this time equals the product of this time and the average of the working metabolism and that of the first determination

3) the still remaining time was multiplied by the metabolic rate of the first determination

3.4. Functional relationships

3.4.1 *Relation between change in mean body temperature and changes in rectal and mean skin temperature*

At the end of the recovery period the subject's body weight was determined for the sixth time (G_6). Dividing the observed heat lost in excess in the recovery period (ΔH_3) by the product of this body weight and the specific heat of humans 0.83 (cited from BURTON and EDHOLM 1955) the change in mean body temperature may be obtained. This temperature change may be related to the changes in rectal and mean skin temperature appearing in the same period. Since the change in rectal temperature is largely determined by the metabolic rate in the preceding period, the experiments with one particular metabolic rate have been pooled and the change in mean body temperature is plotted against the change in mean skin temperature at one averaged change in rectal temperature (Figs. 3.21 and 3.22). Analysis of covariance reveals

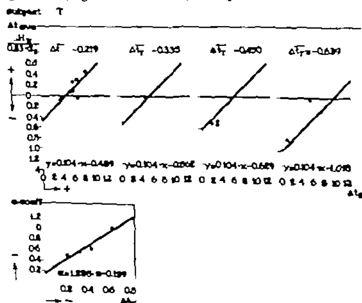


Fig. 3.22.

Subject v T. Change in mean body temperature calculated from the heat lost in excess in the recovery period, as a function of the change in mean skin temperature and the change in rectal temperature both observed in the recovery period.

in Fig 3 20 (mean difference for W.K +2 09 and for ι T -1 13 standard deviations 25 2 and 25 4 respectively Student s t test for differences with double-tailed probability greater than 0 50) The standard deviation of the differences of both subjects combined is given in the figure Assuming the variance to be entirely due to ΔH_1 this figure gives a standard deviation of less than 0 5 kcal per minute for the sum of the three components of the heat balance. The accuracies of two of them are known (for metabolic rate 0 10 kcal/min (section 2 0) and for evaporation heat loss 0 41 g/min (section 3 2 2.?) or about 0 27 kcal/min) so that the standard deviation of the third component must be about 0 3 $(0\ 5)^2-(0\ 1)^2-(0\ 27)^2=(0\ 3)^2$ Actually it will be smaller since the assumption that the variance is entirely due to ΔH_1 is incorrect. The validity of the agreement will be discussed in Chapter 4

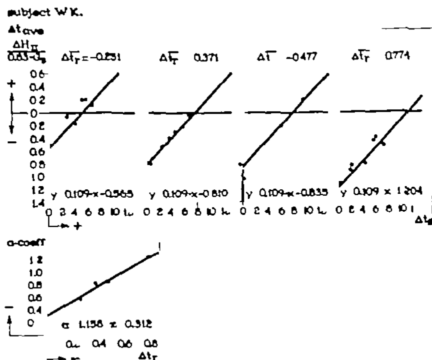


Fig 3.21

Subject W.K. *Change in mean body temperature calculated from the heat lost in excess in the recovery period as function of the change in mean skin temperature and the change in rectal temperature both observed in the recovery period.*

of the heat accumulated in the period of work and heat load. Applying ΔH_2 instead of ΔH_1 to the changes in rectal and mean skin temperatures observed in the period of work and heat load gives the following equations

$$\text{for subject W.K.}\quad \frac{\Delta H_2}{0.83\ G_4} = 0.115\ \Delta t + 0.852\ \Delta t_r + 0.40$$

$$\text{for subject v T}\quad \frac{\Delta H_2}{0.83\ G_4} = 0.094\ \Delta t + 0.771\ \Delta t_r + 0.26$$

In this material the analyses of covariance give insignificant differences in the regression coefficients but highly significant differences in the a-coefficients. They are shown in Table 3.3

TABLE 3.3

Tentative analysis of the contribution of the rectal temperature to the mean internal temperature

Subject W.K.				Subject v T			
$\overline{M}_{gross}$	a-coeff.	Δt_r	$\frac{\Delta t_r}{a\text{-coeff.}}$	$\overline{M}_{gross}$	a-coeff.	Δt_r	$\frac{\Delta t_r}{a\text{-coeff.}}$
2.78	+0.616	+0.282	0.46	3.05	+0.505	+0.213	0.42
4.31	+0.868	+0.466	0.54	4.94	+0.507	+0.403	0.80
5.93	+0.942	+0.736	0.78	6.86	+0.666	+0.677	1.02
8.66	+1.336	+1.066	0.80	8.97	+1.059	+0.923	0.87

together with the average changes in rectal temperature and the average metabolic rate. The results seem to suggest that the rectal temperature more nearly approaches the mean internal temperature (a-coefficients) at higher metabolic rates.

3.4.2. *Change in rectal temperature during exercise as a function of metabolic rate and dehydration*

Subject W.K. shows an effect of preceding dehydration on his final rectal temperature during exercise (Fig. 3.4). Both subjects show a relation between dehydration and the amount by which the rectal temperature remains higher at the end of the recovery period than at the end of the first period of sitting (Figs. 3.13 and 3.14). In order to establish a subdivision of the changes in rectal

that the four regression coefficients of one subject do not differ significantly Thus one common regression coefficient may be calculated for each subject. The lines with this coefficient are shown The y-intercepts where Δt_s is zero differ significantly They indicate the change *in mean body temperature due to change in* rectal temperature only In a separate graph the a coefficients are plotted against the mean change in rectal temperature They show a fairly linear relationship but the line does not pass through the origin The regression coefficients in the main graphs indicate the contribution of the change in mean skin temperature to the change in mean body temperature A surprisingly low percentage of approximately 10 % is found in both subjects A similar small contribution was recently reported by STOLWIJK and HARDY (1966) The contribution of the rectal temperature to the change *in mean body temperature exceeds 100 %* which is *surprising* since a value of around 90 % was to be expected. The general equations for the change in mean body temperature are

for subject W K $\frac{\Delta H_2}{0\ 83\ \ G_s} = 0\ 100\ \Delta t_s + 1\ 158\ \Delta t_r - 0\ 31$

for subject v T $\frac{\Delta H_2}{0\ 83\ \ G_s} = 0\ 104\ \Delta t_s + 1\ 255\ \Delta t_r - 0\ 16$

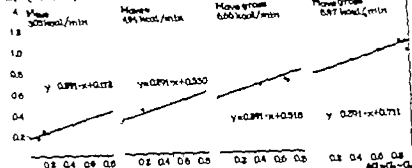
The weighting factors for the change in rectal temperature and the negative constants indicate that change in rectal temperature does not quantitatively represent a change *in internal temperature.* Application of the same technique using the heat accumulated in the period of work and heat load (ΔH_1) and the corresponding changes in rectal and mean skin temperature gives the following equations

for subject W K $\frac{\Delta H_1}{0\ 83\ \ G_4} = 0\ 103\ \ \Delta t_s + 0\ 537\ \ \Delta t_r + 0\ 55$

for subject v T $\frac{\Delta H_1}{0\ 83\ \ G_4} = 0\ 085\ \Delta t_s + 0\ 691\ \ \Delta t_r + 0\ 10$

The weighting factors for the change in mean skin temperature remain almost unaffected In these analyses of covariance however the a-coefficients do not differ significantly (not shown)

From Fig 3 20 and the discussion in section 3 3 3 3 it may be concluded that ΔH_2 with reversed sign is the best available estimate



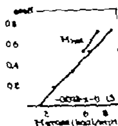
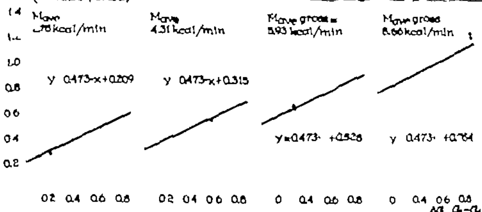


Fig. 3.24.

Subject v T. Changes in rectal temperature as function of dehydration and metabolic rate.

temperature. The lower work loads occurred without inclination of the treadmill. Thus in these cases the gross metabolic rate was equal to net heat production in the body. At the other two work loads there was a certain amount of external work and by consequence the net metabolic heat production was smaller than the gross metabolic rate. This finding is in agreement with the results in Fig. 3.6 section 3.2.2.1. It seems to be the answer to JACKSON and HAMMEL (1963), quoted in the introduction (section 1.1) who doubted that the rise in rectal temperature is caused by a set point shift. Both subjects show almost the same proportionality constant and both lines originate at a metabolism of approximately one kcal/min. The pure effect of dehydration is shown by the common regression coefficients. These figures indicating the rise in rectal temperature due to dehydration alone are individually different, but the coefficients agree reasonably well with those

temperature into a change due to work and a change due to dehydration an analysis of covariance was carried out (Figs. 3.23 and 3 24) Since a change in rectal temperature is known to be



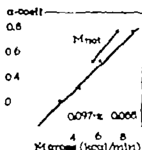


Fig 3.23

Subject W K. Changes in rectal temperature as a function of dehydration and metabolic rate

influenced by the metabolic rate the data at one metabolic rate were pooled and in these four groups the change in rectal temperature was plotted against the weight loss during the exercise period (G_1-G_4) The analysis revealed that the four regression coefficients of one subject do not differ significantly The lines with the common regression coefficient are shown in Figs 3 23 and 3 24 The y intercepts where weight loss is zero (a-coefficients) differ highly significantly On the separate graph these a-coefficients were plotted against gross metabolic rate They show a straight line relationship This linear function indicates the pure effect of metabolic rate on rectal temperature It is noteworthy that metabolic rate rather than net heat production determines the rise in rectal

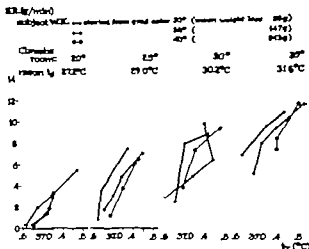


Fig. 3.25.

Subject W.K. Sweating rate as a function of rectal temperature at four different levels of skin temperature.

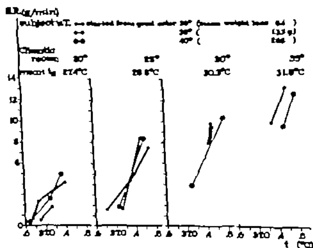


Fig. 3.26.

Subject v T Sweating rate as function of rectal temperature at four different levels of skin temperature.

line to the right except in the range of low sweating rate. As mentioned before the experiments with v T are incomplete He

calculated in Figs. 3.13 and 3.14 although they are a little smaller. This may be attributed to the inequality of heat gains and losses at the end of the recovery period (Fig. 3.12) or in other words to the fact that the rectal temperature did not return to the expected level. Contrary to other groups of data to which the analysis of covariance is applied, in these data the four groups of x values $(G_1 - G_1)$ are not equally distributed over the abscissa. This might considerably influence the a-coefficients. A multiple regression analysis (SNEDECOR, 1950) does not have this disadvantage. For subject W. K. the following equation was obtained:

$$y = 0.450\ x + 0.097\ z - 0.060$$

where $y = \Delta t_r$
$x = \Delta G$
$= M$

The standard error of the first constant in 0.002, that of the second constant 0.02. The agreement of the two equations obtained with both statistical techniques is so close that it may be regarded safe to apply the analysis of covariance to this material.

3.4.3 *Sweating rate as a function of body temperatures and as a function of the change in mean body temperature*

In this section the sweating rate is first plotted as a function of rectal and mean skin temperatures according to the classical concept and secondly as a function of the change in mean body temperature as calculated from the heat accumulated in the body during the period of work and heat load according to Hardy's concept of a type of summation of the combined inputs from the receptors.

3.4.3.1 *Sweating rate as a function of rectal temperature at four levels of mean skin temperature* all observed at the end of the period of work and heat load (Figs. 3.25 and 3.26). The well known increase of sweating rate with increasing rectal temperature may be seen at each skin temperature. The lines show inexplicable bends within one set of experimental conditions. *In subject W. K.* the effect of the preceding period of sitting in the gradient calorimeter, probably the effect of dehydration, may be seen as a shift of the

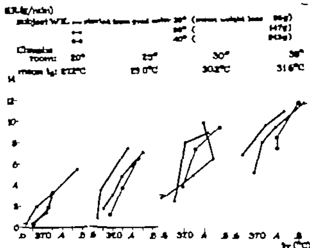


Fig. 3.25.

Subject W.K. Sweating rate as function of rectal temperature at four different levels of skin temperature.

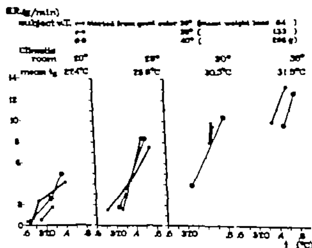


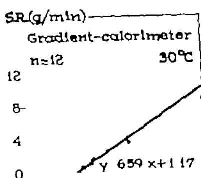
Fig. 3.26.

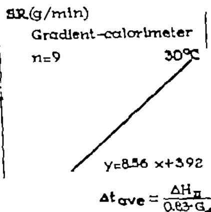
Subject v T Sweating rate as function of rectal temperature at four different levels of skin temperature.

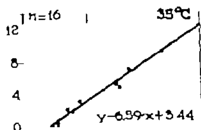
line to the right, except in the range of low sweating rate. As mentioned before the experiments with v T are incomplete He

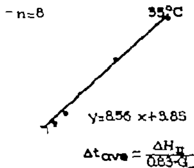
does not show any sign of an effect of the preceding sitting period but the relation between sweating rate and rectal temperature is a band rather than a line In other words the same sweating rate may be present at two rectal temperatures differing not less than 0 ?–0 3°C.

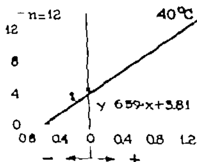
3 4 3 2 *Sweating rate as a function of the change in mean body temperature* In Fig 3 27 the sweating rate observed at the end of the period of work and heat load is plotted against the change in mean body temperature calculated from the heat lost in excess in the recovery period (ΔH) divided by 0 83 G_4 Strictly speaking this ΔH_2 is not the heat accumulated in the period of work and heat load but it may be concluded from Fig 3 20 and the discussion in section 3 3 3 3 that it is the best available estimate Three graphs per subject are obtained one for each gradient-calorimeter temperature or one for each given but unknown initial mean body temperature In subject W K the three regression coefficients do not differ significantly (analysis of covariance) and the common regression coefficient gives three lines (shown in the graphs) with y intercepts which differ highly significantly These sweating rates without any change in mean body temperature show a fair agreement with the averages of the sweating rates observed at the end of the first period of sitting in the gradient calorimeter (indicated by crosses on the zero ordinate) Subject W K sweats 0 50 g/min when his mean body temperature rises 1 C above a critical level A multiple regression analysis using $S R$ $\Delta t_{s\ e}$ and calorimeter temperatures as variables yields 0 52 g/min with a standard error of 0 21 The effect of previous dehydration as seen in Fig 3 25 cannot be detected probably due to the division of ΔH_2 by G_4 a dehydration of 0 85 kg (maximum value observed during the period of work and heat load) affects G_4 in the denominator by less than 2 % The observations at 40 C gradient-calorimeter temperature show a larger scatter than at the other temperatures. This is probably due to the larger variation in the air temperature (Table 3 1) of the gradient calorimeter In subject v T the three regression coefficients do not differ significantly nor do the y-intercepts. The conclusion however that v T sweats 8 50 g/min for a rise of 1 C in mean body temperature remains valid In this material the multiple regression analysis yields 8 32 g/min with a standard error of 0 02.











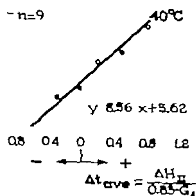


Fig. 3.27
Sweating rate as function of the change in mean body temperature.

4. DISCUSSION

4 1 List of experimental results

The experimental results relevant to the following discussion are listed here briefly

4 1 1 *Derived from thermometry*

4 1 1 1

The rectal temperatures at higher work loads (6 and 7½ kcal/min) do not show a steady state (Fig 3 16)

4 1 1 2

Rectal temperature does not return to its original level the difference being proportional to the weight loss observed during the corresponding time interval One subject W.K has a rise of half a degree Celsius per kg dehydration the other v T one third of a degree per kg (Figs 3 13 and 3 14) An estimate of the same effect observed in the period of work and heat load reveals 0 47°C/kg for W K and 0 29 C/kg for v T (Figs. 3 23 and 3.24)

4 1 1 3

The change in rectal temperature during exercise can be divided into a change due to exercise and a change due to dehydration (Figs. 3 23 and 3 24) The change due to exercise is nearly the same for both subjects (W.K 0 097°C/kcal/min v T 0 092°C/kcal/min) *and is proportional to metabolic rate rather than to net metabolic* heat production. The change due to dehydration is described in 4.1 1 2

4 1 2 *Derived from calorimetry*

4 1 2 1

Both subjects seem to establish a balance between heat gains and heat losses at the end of the first two periods of the experiment (Figs 3 3 3 8 and 3 9) and almost so at the end of the recovery *period (Fig 3 12)*

4.1.2.2

The heat accumulated during the period of work and heat load seems to be equal to the excess heat lost during the recovery period (Fig. 3.20). Thus the more accurate determination of the excess heat loss in the recovery may be used as the best available estimate of the heat accumulation during the period of work and heat load.

4.1.2.3.

Sweating rate increases with increase in mean body temperature and conversely. For each change in mean body temperature of one degree Celsius above a critical level subject W.K. sweats 6.59 g/min more. Subject v.T. has a higher sensitivity sweating 8.58 g/min more for each degree Celsius rise.

4.1.3 *Derived from both thermometry and calorimetry*

4.1.3.1

When analysing changes of mean body temperature in terms of its components change in mean skin temperature and change in rectal temperature, the part of the mean skin temperature is independent of work load and ambient temperature (in the range investigated) and is about 10 % for both subjects. The remaining part may be expressed as a linear function of the change in rectal temperature but this equation contains an inconsistent weighting factor and an inexplicable constant (section 3.4.1., Figs. 3.21 and 3.22). This suggests that a change in rectal temperature is not representative for a change in mean internal body temperature.

4.2. THERMOMETRY VERSUS CALORIMETRY

4.2.1 *Thermal equilibrium*

If no rectal temperature measurements would have been made it would have been easy to assume that thermal balance is present or almost present at the end of each of the three periods of the experiment because heat gains and heat losses are equal within

the accuracy of the measurement (Figs 3 3 3 8 and 3 9 3 1°). Yet rectal temperature seldom reaches a steady state at the end of these periods (Fig 3 10) However an estimation of heat storage from these temperature changes will provide results lying well within the error band of Figs. 3 3 and 3 12 even if the contribution of the rectal temperature is exaggerated by assuming an unrealistic *weighting factor of 100 %* and the slight decreases in mean skin temperatures (Fig 3 15) are neglected A calorimetrical analysis of Fig 1 1 where a continuous rise in rectal temperature is seen is even more surprising The results are shown in Table 4 1 Each

TABLE 4 1

Calorimetrical analysis of Fig 1 1

Period	1		3	4	5	6	7	8
$\dot{M}_{net}$	+7.51	+7.16	+7.0[illegible]	+7.04	+7.47	+7.20	+7.28	+8.69
$(\dot{R}+\dot{C})$	+1.27	+1.61	+1.67	+1.71	+1.69	+1.07	+1.71	+1.64
$\dot{E}$	−7.50	−8.73	−8.60	−8.62	−8.27	−8.08	−7.59	−8.16
$\dot{E}_{req}-\dot{E}$	+1.28	+0.04	+0.09	+0.13	+0.89	+0.70	+1.40	+2.17
Δt in °C	+0.6	+0.14	+0.16	+0.3	+0.15	+0.16	+0.13	+0.46
Δt in °C	+1.75	−0.47	−0.42	+0.33	+0.[illegible]6	+0.10	+0.10	+0.47
ΔH kcal/min	+[illegible].11	+0.[illegible]7	+0.23	+0.94	+0.46	+0.45	+0.37	+1.32

column applies to the corresponding period of 28 8 min The error of the harness and the coefficient for the non-evaporative heat exchange as applied to this subject J V are averages of those obtained from subjects W K and v T (Table 3 2) The heat *storage* (ΔH) *is calculated from* Δt_r *and* Δt using the average of the first pair of equations obtained in 3 4 1 When only $\dot{E}_{req}-\dot{E}$ is considered a thermal balance seems to have been obtained in periods 2 3 and 4 which cover nearly 1½ hours after which sweating rate starts to fail to meet the requirements. On the other hand the heat storage (ΔH) calculated from the change in body temperatures shows a positive figure throughout without any distinct sign of disequilibrium after the 4th period This discrepancy cannot be explained by incorrectly chosen constants such as error of the harness coefficient for the non-evaporative heat exchange or weighting factors for body temperatures since other values for these constants only affect the *numerical value of the figures* but

not the trend with time. The cardinal question is whether a thermal equilibrium exists during the periods 3 and 4 or not. If not, it is not clear why the storage is uniform both in quasi-equilibrium conditions and definite disequilibrium conditions. If yes, the question may be raised why the rectal temperature rises and where in the body the temperature drops in proportion. The author cannot present a satisfactory solution.

4.2.2. $\Delta H_1 = \Delta H_2$ *and rectal temperature remaining higher at the end of the recovery period in proportion to the weight loss*

Results 4.1.1.2 and 4.1.2.2 seem to be contradictory. When ΔH_1 calories are absorbed by a mass M_1 the temperature will rise to a certain level. When the same amount of heat is lost by a smaller mass the temperature should fall more than it has risen. This is not the case in the actual experiments. Either ΔH_1 does not equal ΔH_2 or the change in rectal temperature is not a good measure of the change in mean internal body temperature, or both. The second alternative is confirmed by result 4.1.3.1 but this does not imply that ΔH_1 and ΔH_2 are equal.

4.2.3

Summarizing it can be stated that several results from thermometry and calorimetry are contradictory and several questions remain open. When dehydration is moderate the theoretical inequality of ΔH_1 and ΔH_2 due to loss of body mass cannot be demonstrated, since a loss of one kilogram from a total body mass of an adult man means a change by less than 2 %. Therefore in the experiments presented here ΔH_2 can safely be regarded as the best available estimate of the heat accumulated during the period of work and heat load.

4.3 Change in rectal temperature and change in body weight

There are two processes which have a marked influence on the rectal temperature metabolism during exercise and dehydration. The influence of dehydration on rectal temperature is seen in both

subjects when the rectal temperatures at the end of the recovery period are considered (Figs 3 13 and 3 14) The subject with the largest influence shows an effect even with small dehydrations, ranging from 50 to 250 g (Fig 3 4) It might be objected that rectal temperature has not yet returned to its initial value but it has been demonstrated from the heat balance returning almost to its initial value that initial conditions have been nearly regained. Moreover the fair agreement of the two determinations of $\Delta t_r/\Delta G$ found with different techniques and from data obtained from two different periods of the experiment may also support this conclusion It is more likely that this elevated temperature will not disappear unless the subject restores his lost volume When he does rectal temperature will drop precipitously (SENAY and CHRISTENSEN 1965)

4 4 CHANGE IN RECTAL TEMPERATURE AS A MEASURE OF THE CHANGE IN MEAN INTERNAL BODY TEMPERATURE

It is worthy of note that changes in rectal temperatures can be understood in terms of changes in metabolic rate and dehydration (result 4 1 1 3) but that changes in rectal temperature do not cover quantitatively changes in internal temperature as calculated from changes in mean body temperature (result 4 1 3 1) It is known that other internal temperatures such as oesophageal and tympanic temperatures tend to run more or less parallel to the rectal temperature after an initial phase shift when the subject performs physical exercise The discrepancy between results 4 1 1 3 and 4 1 3 1 mentioned above may suggest that changes in rectal temperature reflect fairly well the changes in what is usually regarded as internal temperature (temperature of the aortic blood?) but that it does not reflect changes in other internal parts of the body such as working muscles (c.f ROBINSON *et al* 1965)

4 5 SWEATING RATE AS A FUNCTION OF THE CHANGE IN MEAN BODY TEMPERATURE

The main objective of this study namely the attempt to establish a functional relationship between the change in mean body temperature and sweating rate has been achieved as seen from Fig 3 27

The results support Hardy's view that thermal sweating is elicited by a "type of summation of the combined inputs from the receptors all over the body surface and in other tissues In this study a representative value has been determined from calorimetrical measurements for expressing overall thermal changes in the body in terms of a temperature instead of a vague summation of the combined inputs" The change of this mean body temperature shows a linear relationship with sweating rate No sign of "saturation of the control system" (WYNDHAM 1965) was detected in the range investigated (up to approx 1 g/min) It is admitted that the subjects were not exerted to near maximal sweating rates where such a saturation might be expected. The two subjects of whom one was much more used to physical exercise than the other show distinct differences in their signal-response curves. The subject with the greatest response (v T) does not show a statistically significant difference in mean body temperature at different gradient-calorimeter temperatures prior to the period of work and heat load. (Actually the sweating rates did not differ significantly at the points corresponding to zero change in mean body temperature.) This might indicate that, due to the high sensitivity of the control system, mean body temperature is maintained at nearly the same level although the subject is exposed to three different calorimeter temperatures.

5 SUMMARY

Thermal sweating is a response to too high body temperatures. The current model of the control mechanism of thermal sweating has superficial (skin) temperatures and internal (rectal, esophageal, tympanic) temperatures as incoming signals and sweating rat as controlled output. Controversy exists concerning the mode of interaction between superficial and internal temperatures. This controversy might be resolved if one could obtain some representative body temperature independent of thermometry In this study a change in mean body temperature has been determined by measuring calorimetrically the heat accumulated during work and heat load and by dividing this amount of heat by the product of body weight and specific heat.

In order t measure the heat accumulated each experiment has three periods first period of sitting in a gradient calorimeter then a period of work and heat load, and finally a period of recovery in the gradient calorimeter again, held at the same temperature as in the first period. Each period lasted approximately one hour The following variables were

measured at regular intervals air and mean skin temperatures, rectal temperature dew point temperature metabolism and weight loss. In the first and third period weight loss and non-evaporative heat exchange were recorded continuously Three calorimeter temperatures and 1 different combinations of work and heat load were chosen and experiments were carried out in all possible combinations. The humidity was low

Two subjects were investigated At the end of each period an equilibrium between heat gains and heat losses seemed to be established The heat accumulated during the period of work and heat load was estimated calorimetrically and appeared to be equal to the excess heat lost during the recovery period which was also determined calorimetrically Most variables investigated including sweating rate returned almost to their original values observed at the end of the first period with the exception of the rectal temperature which remained higher in proportion to the dehydration in the intermediate period A constant ratio of 0.5 C/kg and 0.3°C/kg respectively was observed in the two subjects. The subject with the highest ratio showed a shift upwards of the relation between rectal temperature and metabolic rate during exercise in proportion to the dehydration in the preceding period of sitting in the gradient calorimeter An analysis of the change in rectal temperature during exercise into its component parts, a change due to dehydration and a change due to exercise revealed a dehydration effect of 0 47°C/kg and 0.20°C/kg respectively The change in rectal temperature due to exercise alone was nearly the same for both subjects and turned out to be proportional to metabolic rate rather than to net metabolic heat production

An analysis of the change in mean body temperature into its component parts, change in mean skin temperature and change in rectal temperature revealed a contribution due to the change in skin temperature of approximately 10 % for both subjects, but the remaining change in mean internal temperature was not directly proportional to the change in rectal temperature. A tentative explanation is that changes in rectal temperature reflect with a considerable lag changes in aortic blood temperature but not other internal temperatures such as temperatures in working muscles.

The sweating rate during exercise was directly proportional to the change in mean body temperature 6 59 g/min per degree centigrade and 8.56 g/min per degree centigrade for the two subjects respectively

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APPENDIX

S	d.f.	Calculation	
S	$k-1$	$\sum \frac{C^2_{xyi}}{C_{xxi}} - \frac{C^2_{xyb}}{C_{xxb}}$	
S	$n-2k$	$C_{yyb} - \sum \frac{C^2_{xyi}}{C_{xxi}}$	The test of whether the slopes of the regression lines within the groups are the same
S_b	$n-k-1$	$C_{yyb} - \frac{C^2_{xyb}}{C_{xxb}}$	$H_0(I)$ $F = \frac{(n-2k)}{(k-1)} \frac{S_c}{S_r}$
S	$k-1$	$C_{yyt} - \frac{C^2_{xyb}}{C_{xxb}} - \frac{C^2_{xyT}}{C_{xxT}}$	The test for difference in means $H_0(II)$ $F = \frac{(n-2k)}{(k-1)} \frac{S_t}{S_T}$
S_T	$n-2$	$C_{yyT} - \frac{C^2_{xyT}}{C_{xxT}}$	

Key for the symbols of the different sources of variance and the formulae for the tests of significance.

DATA OF THE ANALYSES OF COVARIANCE

Fig. 3 4.

	1	2	3	4	5	6	T
Cxx	58.3060	81.9040	52.9362		190.1962	1.0222	191.2184
Cxy	7.0306	12.1422	6.8332		26.0060	−0.0802	25.9258
Cyy	1.1046	2.2284	1.0460		4.2960	0.3067	4.5057
$(Cxy)^2/Cxx$	0.8779	1.8000	0.8812		3.5556		3.5151

	d.f.
$S_r = 0.7299$	24
$S_c = 0.0032$	2
$S_b = 0.7331$	26
$S_t = 0.3475$	2
$S_T = 1.0806$	28

$H_0(I)$ $F = 0.08$ $F_{0.05} = 3.32$

common regression coefficient $= 0.136$

$H_0(II)$ $F = 8.06$ $F_{0.01} = 5.35$

Fig 3.8.

	1	-	3	4	b	t	T
Cxx	1 4 08	191 00	183 60	160 97	060.45	38.50	693.95
Cxy	51 ??9	95 400	104 71	70 36	3*7 *6_	110.203	437 447
Cyy	?? 5313	49 8151	00 6870	39._658	17_1098	3 4 65*9	496.835
$(Cxy)^2/Cxx$	21 049	47 4 66	60 181	36.3834	162.16*7		273.903

	d.f
S = 8.1 5	3*
S = 1 0140	3
S_b = 10 0371	35
S_t = 03.7698	3
S_T = ?73 8060	38

$H_0(I)$: F_0 = * 61 $F_{0.05}$ = 2.8

common regression coefficient = 4.09

$H_0(II)$: F_0 = 346.389 $F_{0.05}$ = 3.9

Fig 3.21

	1	2	3	4	b	t	T
Cxx	57 *418	55 4340	87 0390	4_4176	*4_13*4	10.8876	58.0270
Cxy	3 03 14	5 14403	1* 37609	5 14517	6.?0803	7.51689	33.8140
Cyy	0 6 586	0 811396	*_40410	1 047803	4 7 5540	3.517022	8.4298
$(Cxy)^2/Cxx$	0 30460	0 477455	1 750758	0 6 4090	_856*3		4.4163

	d.f
S = 1 033759	3
S = 0 35549	3
S = 1 860308	35
S = 1 058735	3
S_T = 3 828043	38

$H_0(I)$: F_0 = 1.54 $F_{0.05}$ = 2.8

common regression coefficient = 41

$H_0(II)$: F_0 = 1* 79 $F_{0.05}$ = 3.9

Fig 3 23

	1		3	4	b	t	T
Cxx	0 087714	0 18904	0 179109	0 159718	0 61657	1_36430	1.83307
Cxy	0 0*980	0 1_0 7	0 00*4*	0 135 4	0 0180	_06*69	_3345
Cyy	0 30*8	0 4490	0 0500	0 *900	1 0030	3 491*	4.534
$(Cxy)^2/Cxx$	0 0101	0 0881	0 0000	0.1145	0 1381		2.9918

	d f
S = 0 8168	3
S = 0 1381	3
S_b = 0 9549	35
S = 0 6374	3
S_T = _9910	38

$H_0(I)$ F_0 = 0 180 $F_{0.05}$ = 2.8

common regression coefficient = 0.17

$H_0(II)$ F_0 = 8.3* $F_{0.05}$ = 3.9

Fig 3.2 Subject W K

	1		3	4	b	t	T
Cxx	3 _43185	2.451868	1 874*08		7 560351	0 084582	8.___
Cxy	1 8697	18 06503	10 5 858		49 88058	-0.55616	49.3*48
Cyy	160 0557	147 46 6	134 817		431 8000	0 3*03	432.1*0
$(Cxy)^2/Cxx$	139 719	133 1007	60 14 7		3*8 7035		284 418

	d f
S = 99 8374	34
S = 3 2501	2
S = 103 0965	36
S_t = 44 6051	*
S_T = 147 7010	38

$H_0(I)$: F_0 = 0 56 $F_{0.05}$ = 3.2

common regression coefficient = 6.9

$H_0(II)$ F_0 = 7 60 $F_{0.05}$ = 4.2

Fig 3.5.

	1		3	4	b	t	T
Cxx	45.7458	44 7367	56.5039		146.9014	0.3464	147.333
Cxy	6 3750	3.9*67	6.9140		17 157	-0.0161	17 198
Cyy	1 0538	0 4084	1 0453		2.5075	0.0180	_534
$(Cxy)^2/Cxx$	0 8884	0.3447	0.8450		2.0163		_003

	d f
S_r = 0.4 85	*0
S = 0 00*7	2
S_b = 0 491*	
S_t = 0 0?74	
S_T = 0.5185	4

$H_0(I)$: F = 1 46 $F_{0.05}$ = 3.3

common regression coefficient = 0.11

$H_0(II)$: F_0 = 0.04 $F_{0.05}$ = 4.2

Fig. 3.9

	1	2	3	4	5	6	T
Cxx	37.30	7.97	113.78	78.9*	225.97	170.41	418.38
Cxy	17.713	5.294	56.230	50.832	120.039	271.201	401.260
Cyy	8.8071	3.5145	28.1769	32.8932	73.1817	435.4459	505.7375
(Cxy)²/Cxx	8.4115	3.5032	27.7888	32.3310	70.1917		284.8405

	d.f.
= 1.1572	18
= 1.2501	3
S_4 = 2.4073	21
= 121.3097	3
S_T = 123.7070	24

H_0(I) $F = 6.48$ $F_{.95} = 3.16$

common regression coefficient = 0.544

H_0(II) $F = 826.76$ $F_{.95} = 6.03$

Fig. 3.22.

	1	2	3	4	5	6	T
Cxx	12.21	15.12	34.11	28.32	90.77	39.87	130.64
Cxy	1.1174	0.8196	4.6264	3.1448	9.4074	9.2250	18.6420
Cyy	0.220361	0.068459	0.778133	1.048421	2.121574	2.806777	4.930351
(Cxy)²/Cxx	0.102259	0.017858	0.627218	0.337204	0.974969		2.660167

	d.f.
= 1.038937	18
= 0.109634	3
= 1.148591	21
= 1.122593	3
S_T = 2.270184	24

H_0(I) $F = 0.63$ $F_{.95} = 3.16$

common regression coefficient = 0.104

H_0(II) $F_0 = 6.60$ $F_{.95} = 6.03$

Fig. 3.24.

	1	2	3	4	5	6	T
Cxx	0.021062	0.009017	0.107997	0.128027	0.283103	1.676909	1.962012
Cxy	0.02242	0.01098	0.06882	−0.01938	0.08238	1.91373	1.99659
Cyy	0.1696	0.0621	0.1361	0.2310	0.4588	2.1994	2.7882
(Cxy)²/Cxx	0.0162	0.0150	0.0438	0.0027	0.0241		2.0318

	d.f.
S_1 = 0.4111	18
S_2 = 0.0336	3
S_3 = 0.4447	21
S = 0.1017	3
S_T = 0.7564	24

H_0(I) $F = 0.283$ $F_{.95} = 3.16$

common regression coefficient = 0.291

H_0(II) $F = 2.25$ $F_{.95} = 6.03$

Fig. 3.27 Subject T

	1	2	3	4	5	6	T
Cxx	1.273780	1.780485	1.618376		4.572851	0.321963	4.894834
Cxy	11.44235	15.36633	12.34456		39.15344	0.60619	39.76163
Cyy	127.1316	153.4953	147.2072		427.8940	4.5468	432.4398
(Cxy)²/Cxx	102.7901	132.6171	100.2626		335.2321		323.0042

	d.f.
S_1 = 92.1342	20
S_2 = 0.5175	2
S = 92.6417	22
S = 16.7839	2
S_T = 109.4256	24

H_0(I) $F = 0.054$ $F_{.95} = 3.49$

common regression coefficient = 8.68

H_0(II) $F = 1.82$ $F_{.95} = 6.09$

Subject W.K. Analysis of covariance given in section 3.4.1

	1	2	3	4	5	6	T
Cxx	80.2266	47.2620	80.8099	44.8725	222.8712	7.0600	230.0312
Cxy	2.99256	4.14091	11.91227	6.63871	25.68545	5.13990	30.82535
Cyy	0.618166	0.716123	2.228484	1.227850	4.790617	3.763216	8.553933
(Cxy)²/Cxx	0.178299	0.362810	1.756295	0.866606	2.950945		4.130754

	d.f.
S_1 = 1.806645	32
S = 0.325104	3
S_3 = 1.831749	35
S_4 = 2.591430	3
S_T = 4.423179	38

H_0(I) $F_0 = 2.30$ $F_{.95} = 2.92$

common regression coefficient = 0.115

H_0(II): $F = 18.347$ $F_{.95} = 5.24$

Subject × T Analysis of covariance given in section 3.4.1

	1	[illegible]	3	4	5	t	T
Cxx	15.03	16.08	3[illegible].6[illegible]	39.38	104.01	1.10	1[illegible]3.11
Cxy	1.3388	0.5306	4.3957	3.4640	9.7300	6.0953	15.8[illegible]
Cyy	0.335587	0.068367	0.760847	1.04010[illegible]	2[illegible]13903	[illegible]510118	4.[illegible]
$(Cxy)^2/Cxx$	0.119254	0.016580	0.592341	0.305 10	0.010[illegible]9		2.0173

	d.f		
$S_r = 1.180518$	18	$H_0(\mathrm{I})$: $F_0 = 0.6[illegible]6$	$F_{[illegible]} = 3.11$
$S = 0.1[illegible]3156$	3		
$S_b = 1.303674$	21	common regression coefficient = 0.[illegible]	
$S = 1.418588$	3		
$S_T = [illegible]7[illegible] 0[illegible]$	[illegible]4	$H_0(\mathrm{II})$: $F_0 = 7[illegible]1$	$F_{[illegible]} = [illegible]$

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TRACER DILUTION CURVES IN CARDIOLOGY AND RANDOM WALK AND LOGNORMAL DISTRIBUTIONS

BY

M. E. WISE

1. INTRODUCTION

Tracer dilution curves ought by this time to have become as useful in circulation research as they are for determining cardiac outputs as a routine. This has not yet happened although the experimental and measurement techniques have become more and more accurate and convenient. For example, the complete curve must still be obtained by extrapolation in order to determine its area and estimate the cardiac output. This extrapolation is unreliable in many cases more often still it is good enough for ordinary routine but not for detecting small changes when much greater accuracy is needed.

From every point of view we want to be able to fit a mathematical function to as much of the primary dye-dilution curve as possible Then we would be more confident both about the usual routine extrapolation of the curve, and of interpreting its form physiologically It has been known for some time that both log normal and random walk distributions provide good fits to dye-dilution curves, except possibly in the upper tail produced by the slowest injected particles of tracer that have passed through the heart only once. This was first proposed by SHEPPARD and SAVAGE (1951), and SHEPPARD (1954 1959 1962) has considered possible random walk models and has produced convincing arguments for interpreting tracer-dilution curves in this way

Recently some geometrical properties of the lognormal distribution have been found to provide a quick and accurate method of fitting them to most of a typical dye-dilution curve including the whole of the ascending limb and without involving this upper

tail (Wise 1966). On the other hand most of a typical random walk curve can be fitted very closely by a lognormal curve, and vice versa. In this paper we shall show how this is done, so that observed curves can be readily fitted by a random walk function.

We shall also show how the parameters of the random walk curve may be interpreted in terms of a schematic physiological model. This is illustrated by some results from analysing dye-dilution curves obtained from patients at rest.

Of the usual routine calculations that for cardiac output proves to be simple enough, but care is needed in defining the distribution of passage times between injection and place of measurement, and *great care is needed over the so-called central blood volume* according to the random walk model.

2. THE EXTRAPOLATION PROBLEM

In the curve shown in Fig. 1 the secondary maximum is rather high, but it is very rare that this is completely absent. This curve was one of a large series obtained from dogs in routine testing

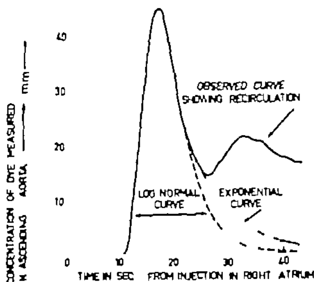


Fig. 1

A dye-dilution curve obtained from routine testing of anaesthetics on dogs at the Janssen laboratories, Beerse, Belgium, showing early recirculation, and a considerable difference between semi-log and lognormal extrapolations of the primary curve.

of anaesthetics [1]). Its form is typical enough. When a dye has passed through the heart and the lungs the concentration time curve measured at a fixed point in an artery or capillary is always positively skew and the upper tail is always masked by the secondary curve. In this example the first recirculating particles, which must have passed twice through at least the left heart, are surprisingly early (That is to say the time of return from the aorta to the heart must be very short.) The consequence is that two plausible methods of extrapolating this primary curve are appreciably different from one another

The exponential extrapolation introduced by HAMILTON (see e.g KINSMAN MOORE and HAMILTON 1929) is used in the great majority of cases. When the logarithm of the ordinate of a dye-dilution curve is plotted against time there is always a straight portion and this is simply assumed to continue into the unobservable part of the primary curve. Also when nearly the whole of the descending limb can be observed, the straight portion is considerable. In many series of experiments comparing the dye-dilution estimates of cardiac output obtained in this way with the direct Fick ones agreement is good, although the standard error of the ratio of the two estimates can be as much as 0 1 (see e.g. HEGGLIN and RUTISHAUSER, 1962 for many other references)

In many other cases, however the straight portion is not long enough to provide a reliable extrapolation. This is particularly liable to happen with many abnormal heart conditions, just when an accurate determination is particularly needed. Then the extrapolation must be obtained somehow or other from the rest of the curve In all cases the form of the descending limb ought to follow consistently from what precedes it.

3. ATTEMPTS TO DETERMINE THE FORM OF A DYE DILUTION CURVE

The most extensive published analysis of actual curves is the one given by Dow (1955) It was adequate to throw doubt on many empirical laws that had previously been proposed. He tried,

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[1]) In the Janssen laboratories, Beerse, Belgium, to whom many thanks for sending a large number of tracings.

among many other things, a logarithmic transformation of the time scale and he gives a very large number of plots of dye concentration against log t when t was the time from the start of the injection Nearly all the curves plotted *in this way were positively* skew but less so of course than the original ones. Most of the curve shown in Fig 1 (in the present paper) does in fact fit extremely well to a lognormal one but the zero of the time has to be made about 7 sec in other words log $(t-7)$ is normally distributed. An approximate calculation based on Dow s plots of concentration against log t showed that if the abscissa scale had been transformed to log $(t-t_0)$ with t_0 equal to about two thirds of the so-called *appearance time then most of his* curves would have become symmetrical

The fact that a lognormal distribution with a zero later than the time of injection, could—sometimes at least—provide an extremely good fit was not easy to recognise until continuous recordings of dye concentrations could be made all Dow s analyses were based on measurements at discrete intervals STOW and HETZEL (1955) gave some examples of good fits to observed curves but the success with this simple distribution has attracted little attention because as Dow pointed out, no definite meaning had been ascribed to the lognormal parameters 'in terms of the hydraulic factors involved in the dispersion of indicator The need to choose an apparently arbitrary zero for the time scale increases this difficulty and it certainly made it much harder to find a simple way of fitting an observed curve The method used by STOW and HETZEL and also by LEWI (1964) depends on estimating the mode accurately which is very difficult in practice

The method of fitting the curve is illustrated in Fig 2. Its main advantage is that the inflection tangents and the maximum can almost always be estimated accurately whilst less reliable variables, such as the mode the appearance time and the slope corresponding to a small part of the descending limb when log y is plotted against t are not used The fits achieved of lognormal to observed curves are so successful in so many cases that this must outweigh any objections *But this success introduces a new difficulty as* soon as we look for a physiological interpretation. The lognormal curve is always regarded as a probability curve Several other skew probability curves are close to lognormal ones except in the extreme

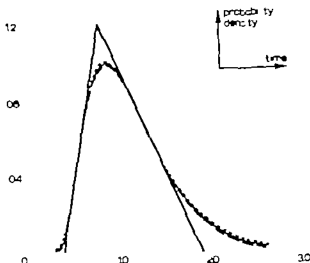


Fig. 2.
Three curves with the same inflection triangle, as given in Table 1
——— random walk function with unit area
------- lognormal function
— — — — gamma function.

upper tail in other words at long times after injection, in the unobservable part. These include the "random walk" curve studied by Sheppard, and other random walk curves. So we are still left with an extrapolation problem.

At least the physiological objection to lognormal curves or similar ones can be answered. The lognormal parameters are indeed, not related to the hydraulic processes because as suggested by Sheppard, the form of the curve is not determined by hydraulic processes but by stochastic processes. Each separate dye particle on its way through the system, must, in effect make many random choices between different pathways It is possible, too that every pulse pressure wave gives rise to some turbulent mixing

4. THE USE OF THE INFLECTION TRIANGLES OF RANDOM WALK CURVES AND THE COMPARISON WITH LOGNORMAL CURVES

The random walk function considered in detail is the "local density" one It is the same as the local density time curve for

flow along a straight tube (see e.g. TAYLOR, 1954). If M particles of tracer are injected quickly at $t = 0$ and the speed of flow is l the number of particles per unit length in a small interval of length at a fixed point along the tube is

$$\frac{M y(t)}{l} = \frac{M}{\mu l} e^{\lambda} \left(\frac{\lambda}{2\pi}\right)^{\frac{1}{2}} \left(\frac{\mu}{t}\right)^{\frac{3}{2}} \exp\left\{-\tfrac{1}{2}\lambda\left(\frac{t}{\mu} + \frac{\mu}{t}\right)\right\} \qquad (4.1)$$

Here $y(t)$ is a probability function and λ and μ are parameters which are defined precisely in section 5. (The derivation of (4.1) is given in Appendix A.) Two other random walk probability functions are important in this problem and these two will be considered later. It has been suggested more than once that this one could be relevant for fitting to dye-dilution curves (KORNER 1961, LEWI 1964). So we shall give the method of applying it in practice and its relation to the lognormal distribution before going into the theory.

The geometrical properties shown in Fig. 2 have proved a godsend in enabling these curves to be analysed quickly. We do not have to wait until on line computers are available in every cardiological unit!

Essentially we profit from the fact that a great deal of the rising limb is very close to a straight line, the tangent at the first point of inflection, and that much of the top half of the falling limb is close to the tangent at the second point of inflection. Also these tangents intersect at a *height that has a nearly constant ratio* to the maximum of the curve; this height ratio decreases very slowly for a considerable increase in the ratio of the slopes of the two inflection tangents. This provides an immediate check on whether *a good fit is possible*.

Fig. 2 and Table 1 give a lognormal, a random walk and a gamma curve for the same inflection triangle. It is clearly difficult to distinguish between them except in the upper tail, but in most dye-dilution curves *this tail is hidden by the secondary maximum*.

THOMPSON *et al.* (1964) showed a most successful fit of a gamma curve to a moderately skew dye-dilution curve and reported many other successes. They also showed a much more skew curve. Again the gamma curve was close to it, but the shape of the rising limb was clearly different. In the gamma curve almost the whole of the rising limb was convex (upwards). (Cf. WISE 1960, par. 7.)

TABLE 1

Local density random walk, lognormal and gamma curves with the same inflection triangle

Local density $y = e^{\lambda} (\lambda/2\pi)^{\frac{1}{2}} t^{-\frac{3}{2}} \exp(-\frac{1}{2}\lambda(t+1/t))$ with $\lambda = 6$

Log normal $t = t_0 + t_L e^{\sigma u}$

$y = y_M \exp(-\frac{1}{2}u^2)$,

(the median is $m + t_0$ where $m = t_L \exp(\sigma^2)$)

with $t_0 = 0.041118$; $t_L = 0.877456$;

$y_M = 1.206009$ $\sigma = 0.452411$

Gamma $y = A e^{-bx} x^{\alpha}$ $x = x_0 + ct$

with $\alpha = 2.87608$

$x_0 = -2.418351$

$c = 8.826772$

$A = 0.1140233$

Ordinates

Time t	Log normal	Random walk	Gamma	Time t	Log normal	Random walk	Gamma
0.2	0.00028	0.00015	0	1.2	0.8075	0.8072	0.8060
0.25	0.00312	0.00228	0	1.3	0.6963	0.6963	0.6963
0.3	0.01842	0.01329	0.00149	1.4	0.5868	0.5862	0.5852
0.35	0.04739	0.04418	0.03617	1.5	0.4883	0.4839	0.4809
0.4	0.10851	0.10384	0.0888	1.6	0.3978	0.3933	0.3875
0.45	0.1958	0.1939	0.1661	1.7	0.3219	0.3157	0.3071
0.5	0.3090	0.3064	0.3063	1.8	0.2567	0.2507	0.2401
0.6	0.6672	0.6669	0.6632	1.9	0.2068	0.1973	0.1854
0.7	0.7970	0.7942	0.7848	2.0	0.1648	0.1548	0.1416
0.75	0.8830	0.8788	0.8661	2.1	0.1306	0.1197	0.1070
0.8	0.9455	0.9404	0.9258	2.2	0.10349	0.09248	0.08028
0.85	0.9846	0.9790	0.9641	2.3	0.08188	0.07109	0.05873
0.9	1.0028	0.9967	0.9823	2.4	0.06474	0.05443	0.04413
0.95	1.0006	0.9947	0.9827	2.5	0.05119	0.04128	0.03242
1.0	0.9799	0.9772	0.9677	2.6	0.04048	0.03160	0.02364
1.05	0.9498	0.9469	0.9402	2.8	0.02533	0.01732	0.01241
1.1	0.9065	0.9067	0.9031	3.0	0.01597	0.01033	0.00639

In this respect the lognormal and random walk curves seem to be more successful even when they are very skew

We have analysed more than 200 dye-dilution curves by fitting them to lognormal curves. All four defining parameters were obtained in each case and others of physiological and statistical interest were deduced from them. (These results will be discussed in another paper).

The physiological interpretation is in terms of random walk curves. The mathematical formulae relating to the random walk curves and their inflection triangles are much more complex than those for lognormal curves (see Appendix B) and we have only

TABLE 2

The inflection triangle of the local density random walk curve
b_1/b_2 ratio of the slopes of the inflection tangents
y_H/y_M height ratio vertex of inflection triangle/maximum of curve
A/Δ area ratio area under complete curve/area of triangle
Parameter λ and n as in (4 1) $1/\lambda = n(n+1)/120$ or

$$n = -1 + \sqrt{(1 + 480/\lambda)}$$

b_1/b_2	y_H/y_M	A/Δ	$1/\lambda$	n
1	1 130	1 033	0	0
1 5	1.2120	1 0436	0 03087	1 7437
2	1 1 0	1 06*7	0 11320	3 194
*.5	1 2000	1 0837	0 10131	4.3174
3	1 069	1 1049	0 6780	5 1916
3.5	1 034	1 1 50	0 34150	5 0210
4	1 1994	1 140	0 41231	0.5518
4.5	1 1952	1 1667	0 48066	7 1112
5	1 1908	1 1871	0.5470*	7 6174

TABLE 3

The parameters of the inflection triangle for the random walk curve (4 1) with $\mu = 1$. From the two columns on the right the values of μ and t_0 (the lower bound) for an observed curve can be estimated

λ	n	T_1	T_H	T_2	Y_H	$T_2 - T_H$	$T_2 + T_1$
∞	0	1	1	1	–	0	2
60	1	0 75903	0 9834	1 7312	3 75670	0.28000	2 03306
20	2	0 60974	0 95084	1 48771	2 17730	0 53687	2 09745
10	3	0 48525	0 90301	1 70454	1.54850	0 80003	2 18979
6	4	0 38375	0 84368	1 92008	1 20801	1 07730	.30474
4	5	0 30298	0 77445	2 13 20	0 99409	1.35775	.43518
20/7	6	0.24034	0 69031	2 33204	0 84738	1 63333	.57298
15/7	7	0 19220	0 62298	2.51645	0 74003	1 80347	2 70874
5/3	8	0 15568	0.54021	67772	0 65874	2 1 851	2 83341

If the observed times are t_1, t_2 and t_H then $\mu = (t_2 - t_H)/(T_2 - T_H)$ and $2t_0 = t_1 + t_2 - \mu(T_1 + T_2)$

The inflection tangents meet the base line at times T_1 and T_2, and meet one another at time T_H height Y_H

numerical results for a few selected values of the shape parameter (This parameter is λ in (4 1) But these should be sufficient, by interpolating for relating the lognormal parameters to random walk ones in all cases that arise at all often in practice

From Tables 2 and 3 the random walk parameters can be obtained directly From Table 2 the height ratio test can be carried out This is if anything even easier than for a lognormal curve The height ratios remain slightly closer to the limiting one of 1 213 and are close to 1.21 for a wide range of slope ratios b_1/b_2 The extrapolated area under the complete curve needed for determining the cardiac outputs can again be obtained as a routine without determining the other parameters the area ratio A/Δ (ratio of area A under the curve to the area Δ of the triangle) varies nearly enough linearly with the slope ratio b_1/b_2, as for a lognormal curve The ratios are slightly less than those for lognormal curves they are much closer to those for the distribution (7 1) which have already been calculated in a few cases. (WISE, *loc cit.*, section 8).

The parameter λ is determined uniquely by the slope ratio When this is greater than 1.5 linear interpolation of $1/\lambda$ seems accurate enough for fitting to observed curves (until fuller tables are available).

The parameters involving time can be obtained from Table 3. The parameter μ – the time taken by the median particle to reach the place of measurement – is best obtained from the *observed* value of $t_2 - t_M$ where the second inflection tangent meets the first one at time t_M and the base line at time t_2 The corresponding theoretical values of $T_2 - T_M$ in the table vary nearly enough linearly with n which in terms of λ is given by $2n = -1 + \{1 + (480/\lambda)\}^{\frac{1}{2}}$

In Table 4 the random walk and lognormal parameters are compared. In nearly all the dye-dilution curves analysed so far the standard deviation σ (lognormal) has been between 0.2 and 0.55 corresponding to slope ratios between 1.5 and 3. Then the random walk parameter λ varies between about 20 and 4 σ varies nearly enough linearly with n (where as before $\lambda = 120/(n^2+n)$). Over this range σ^2 is approximately equal to $1/\lambda$.

The lower bounds agree quite well. The lognormal m – the median is $m + t_0$ – corresponds fairly well with the random walk parameter μ but tends to diverge from it when the curves become

TABLE 4

Parameters of the lognormal curve with the same inflection triangle as the random walk local-density curve. For random walk curve (4.1) $\mu = 1$ and the lower bound is zero. $\log_e (t - t_0)$ is normally distributed with median m and standard deviation σ

Random walk param.		log normal parameters						Random walk	
λ	n	σ	t_0	m	$1/(m\sigma^2)$	t	var t	mean $1+\lambda^{-1}$	variance $\lambda^{-1}+2\lambda^{-2}$
∞	0	0	0	1	∞	1	0	1 1	0
60	1	0.12863	0.00549	1.00296	59.24	1.01685	0.01733	1.01667	0.01722
20	2	0.22630	0.01543	1.01073	19.3_	1.05_58	0.05699	1.05	0.055
10	3	0.32337	0.02780	1.02454	9.334	1.10733	0.1 847	1.1	0.102
6	4	0.42241	0.04112	1.04886	5.343	1.18785	0.23687	1.16667	0.22223
4	5	0.52325	0.05217	1.08908	3.354	1.3010*	0.49118	1.25	0.375
20/7	6	0.62468	0.06096	1.15788	2.213	1.47307	0.97174	1.35	0.595
15/7	7	0.7248*	0.06548	1.22600	1.553	1.65978	1.75658	1.46667	0.90*22
5/3	8	0.82147	0.06643	1.32849	1.115	1.92823	3.34040	1.6	1.32

* See Eq (6.3)

skew The means and variances behave similarly but the variances of skew lognormal curves are considerably larger than the corresponding random walk variances.

A point worth noting is that the random walk median is not equal to the time corresponding to the median particle but is appreciably greater There seems to be no simple way of obtaining it It is considerably nearer to the lognormal median $m+t_0$ than to unity

5. THE RANDOM WALK MODEL IN DETAIL

As originally formulated a particle starts at zero and we consider its distribution of distance from the origin when it has taken a given number of steps n. It has a probability p of moving a unit distance to the right and q of moving the same distance to the left, and $p>q$ The distribution of its distance after n steps is that of a sample of n from this simple (binomial) discrete distribution, and its variance is exactly n times that of a single sample (in fact this equals npq). For most forms of basic distribution the distribution of a sum of n is near enough Gaussian. So we are led to consider a Gaussian distribution with variance increasing continuously as its mean $\bar{x}$ moves to the right. In fact many different stochastic processes seem to yield this simple model as a limiting case It is essential that the different steps, whether few or many should be statistically independent, and it is the variances that are additive and so proportional to the number of steps, or in the simple limiting case to the time taken by the median particle or the distance this particle has moved.

In Fig. 3 for simplicity only the inflection triangles have been drawn. For a normal curve with mean $\bar{x}$ and standard deviation σ_x, the base of the inflection triangle extends from $\bar{x}-2\sigma$ to $\bar{x}+2\sigma$ the height y_H of the vertex is $0\ 48394/\sigma$

The triangles in the lower half of the figure represent Gaussian curves with variances proportional to the distance of the mean $\bar{x}$ from the origin. This is the basis of the random walk model in which the injected particles move in a stream to the right (say) and spread out relative to the position of the median particle which is carried with the stream.

Suppose the variance $\sigma_x^2=k\bar{x}$ when the median particle has

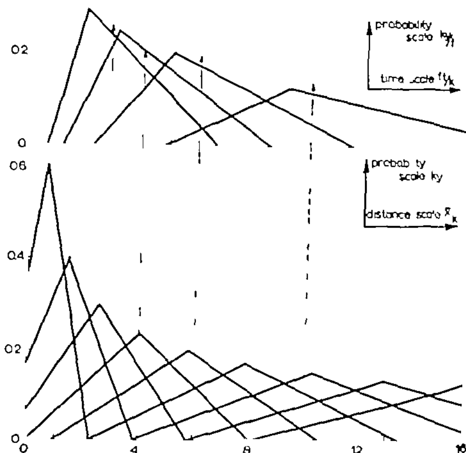


Fig 3

The simple random walk model.

Lower half: inflection triangles for Gaussian distributions of distances of injected particles from the origin Notations as in section 5

Top half Local density dye-dilution curves. At the time represented by each arrow the median particle has reached the place of measurement which is below the arrow on the bottom scale For the triangles shown $\lambda =$ 0/7 4 6 and 10

moved a distance $\bar{x}$ to the right The figure shows the (inflection *triangles of the) distributions obtained when* $\lambda = 1$ *If the* abscissa scale is x/λ it represents all possible cases, provided the ordinate *scale is adjusted so that the area under each curve (in units of* x) equals unity

The *inflection triangles for the corresponding dye-dilution curves* are shown in the top half The arrows give the corresponding place of measurement For example *for unit flow* f *and unit rate of*

spreading out k, for $\lambda = 4$, $\mu = 4$. The time taken for the median particle to reach the place of measurement is μ; the inflection triangles are for curves for the distribution of $\mu t/k$ and every possible dye-dilution curve according to this model is represented on the figure.

Now we consider the actual situation in the human body. This must differ from the model in two major respects. First, the rate of increase of the variance of the normal curves is certainly not constant as the dye moves from a vein through the heart and the lungs to an artery. The lower bounds t_0 for lognormal fits are almost always considerably later than the time of injection. (In very many cases $t_0 \doteq \frac{2}{3} t_1$, where t_1 is the time at the intercept of the first inflection tangent; t_1 is slightly later than the so-called appearance time, which cannot be defined precisely.) The same must hold good for random walk curves. This was also found by Sheppard in relation to fits of dye-dilution curves to first passage time distributions.

In fact this does not upset the model at all, provided the measurements are made in an artery, or a capillary.

The first particles to arrive at this place of measurement belong to Gaussian curves whose median is not far to the left, and the last ones belong to ones with medians rather further to the right of the place of measurement. For example, for measurement at $x/k = 4$ in the figure, the Gaussian curves that influence the dye-dilution curve range from those shown by the 2nd to the 6th triangles in the figure, i.e. from $\sigma = 1.2$ to $\sigma = 3.2$ or $t/k = 1.44$ to 10.24. It is only necessary that the variances σ^2 should increase linearly between these two distances. We can define the zero as an extrapolated one for this linear variation. The analysis of dye-dilution curves shows that in effect, the rate of spreading out must be slower in the heart and/or lungs than near the place of measurement in an artery or capillary.

The second major difference between the model and reality is related to the assumed uniform rate of flow. This is certainly not realistic, either for measurements in the ear, for example, or in the aorta. But it becomes valid if we replace linear flow by volume flow, and appeal to the principle of continuity.

We can consider all the particles of dye that leave the left ventricle at the same instant, and consider the median particles

for the various routes. At time t after leaving the left heart these will have reached various places and the volume they have swept out will equal Qt where Q is the cardiac output provided this is constant. The central blood volume is in fact defined in just this way. We can still think of a linear flow and a variable area of cross section; if the volume flow is constant and the Gaussian distributions of distance are replaced by volume distributions, everything remains valid. More precisely each small interval of distance in the bottom scale of Fig. 2 can be regarded as a real distance multiplied by an equivalent cross sectional area A_e such that $Q = fA_e$.

This area could be the actual one in the aortic valve just outside the left ventricle and then the distance scale further to the right could be regarded as standardised on this area.

6 THE ASYMMETRY IN RELATION TO THE PLACES OF INJECTION AND MEASUREMENT AND THE RATE OF FLOW

If we define the asymmetry in terms of the slope ratio, this depends *only on the parameter λ for a random walk curve* (or σ for a lognormal one). It is clear from the figure that the curves become more symmetrical as one moves to the right. In the same subject *under similar conditions, or in different subjects* with the same rate of spread k for the same place of injection dye-dilution curves obtained from ear measurements should be more symmetrical than one obtained by sampling in the *ascending aorta*.

Similarly for measurements in the same place in an artery or capillary, injections in a vein in an arm, the right atrium, the pulmonary artery and the left ventricle will yield curves in *increasing* order of asymmetry. This agrees in general with what is observed (Dow 1955, Edwards and Korner 1958, Korner 1961).

It does not agree when we compare curves obtained on the same subject before and after exercise. Since $\lambda = f\mu/k = \bar{x}/k$ this must be independent of the rate of flow f if k is the same both before and after exercise. So we try redefining k in terms of a variance proportional to the time from the origin instead of to the distance from the origin, in other words we put

$$\sigma_x^2 = k\mu(x) \qquad (6.1)$$

when $\mu(x)$ is the time taken by the median particle to reach the place of measurement. Then $h = kf$ For any one curve f is assumed to be constant so the form of the dye-dilution curve is not affected We now have

$$\lambda = \frac{f^2\mu(x)}{h} = \frac{f\bar{x}}{h} \qquad (6\ 2)$$

or for lognormal curves that are not too skew

$$f^2 = \frac{h}{m\sigma^2} \qquad (6.3)$$

For large Table 4 shows that $1/(m\sigma^2)$ is too small; its ratio to the corresponding value of $\lambda/\mu(x)$ is that of λ to $1/(m\sigma^2)$ in this table In the detailed analysis $(m\sigma^2)^{-1}$ is involved, so that the error is small when σ is less than about 0.5.

So if the ratio of the variance of the distance distribution of the injected particles to the time from the (extrapolated) origin is more or less constant for one person under varying conditions (or possibly even for different people under comparable conditions), the dye-dilution curves will become more symmetrical with increase in flow for the same places of injection and measurement (constant $\bar{x}$, increase in f) and they will also become more symmetrical for the same flow and increasing "equivalent distances" from place of injection to place of measurement (constant f increasing $\bar{x}$) At any rate this is qualitatively in the right direction.

A distance variance proportional to a time taken rather than a distance travelled by the injected particles, suggests that pulsatile flow plays a role in spreading out the injected tracer while it is passing the place of measurement in an artery or capillary For the normal range of pulse frequencies we could consider the variance of the distribution of distances travelled during the time of a single pulse If this variance was constant, and if no other process was contributing to random mixing – which of course is unlikely – k in (6.1) would in fact be proportional to the pulse frequency Perhaps this helps to illustrate an abstract model physiologically

Whether or no the pulse frequency is important, these relationships between the parameters provide a simple way of testing the model. So far all the numerical analyses have been in terms of lognormal curves, so that (6.3) must be used. In terms of the cardiac

output Q and the equivalent area of cross section A_e, we have

$$(m\sigma^2)^{-\frac{1}{2}} = Q/(A_e l/h) \qquad (6.4)$$

or if we put $h = HP$ where P is the pulse frequency then H has the dimensions of a square of a length and

$$P^{\frac{1}{2}}\,(m\sigma^2)^{-\frac{1}{2}} = Q/(A_e l/H) \qquad (6.5)$$

This can be tested from curves with cardiac outputs ranging as widely as possible but obtained under comparable conditions

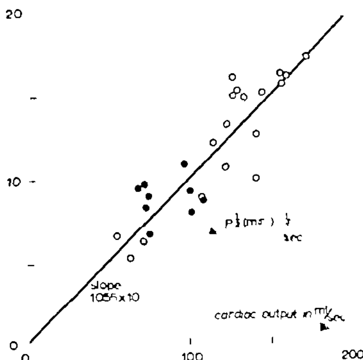


Fig 4

The relationship between the lognormal parameters m and σ the pulse frequency P and the cardiac output Q (all given in Table 5) as predicted in (6.5) according to the random walk model for dye-dilution curves obtained from patients at rest.

Curves obtained from the vegetative physiology laboratory Groningen by Dr F ten Hoor and Prof W G Zijlstra:

- ○ Injections in the pulmonary artery
- + Injection in vena cava superior
- ● Curves from the pneumoconiosis research unit Penarth (Wales) injections in the brachial vein (see COTES, PISA and THOMAS, 1963)

All measurements in the brachial artery

for the patients. On the other hand the sites of injection and measurements need not to be the same. The constants A and H or h cannot depend on the site of injection and H or h should only vary rather slowly if at all with the place of measurement.

Fig 4 shows the results from 28 curves from patients at rest. 19 of them were supplied from a Groningen (Holland) collection of normal curves (without evidence of shunts). Some very low and some very high cardiac outputs were deliberately included.

All curves fitted well to lognormal distributions (and they would fit random walk distributions equally well) the most important parameters are given in Table 5 Both $(m\sigma^2)^{-\frac{1}{2}}$ and $P^{\frac{1}{2}}(m\sigma)^{-\frac{1}{2}}$ when plotted against the cardiac output Q appear to lie about a straight line through the origin. The correlation is rather better when the pulse frequency is included as in (6 5) but not conclusively so [1])

The cardiac outputs from the random walk extrapolation were plotted The varying differences between these and the semi log estimates are quite typical.

The gap in the cardiac outputs is neatly filled by a series from Penarth Wales with a different site of injection. This was part of a series obtained from pneumoconiosis patients. They breathed oxygen and air alternately first at rest and then during exercise. Only the series from the patients who first breathed air at rest is included This fitted the linear relationship convincingly In Fig 4 the units have been reduced to centimeters and seconds since the time parameters of the dye-dilution curves are in seconds e.g. log $(t-t_0)$ is normally distributed with median m and variance σ^2 so that m is in seconds and σ^2 is dimensionless. The slope corresponds to $1/A_{\frac{1}{2}}H$ and has therefore the dimensions of a reciprocal of a volume

The same analysis was tried on curves obtained from a much larger series of patients at rest in the cardiovascular research unit, Hammersmith London, with injections in the vena cava superior and measurements in (just outside) the lobe of an ear (THOMAS *et al.* 1965). Th points shown in Fig. 5 lie about the same straight line but with a much wider scatter For the largest cardiac outputs the points are well below the line but the overall average – of

[1]) If (6.2) is being tested instead of (6.3) the ordinates for the largest values of corresponding to the few very slow dye-dilution curves, will be slightly higher The correlation is as good in this case as the one shown.

TABLE 5

Lognormal parameters for dye-dilution curves from Penarth and Groningen as in Fig 4

t_0 (sec) lower bound

m (sec) σ mean and standard deviation of $\log_e (t - t_0)$

P (min^{-1}) pulse frequency

Q_{sl} Q_{rw} (l/min) cardiac outputs, semilog and random walk extrapolations.

The numbers correspond to different patients curves with the same numbers were obtained at one sitting

Penarth curves

Injections in the left brachial vein about 20 cm proximal to the elbow measurements in the left brachial artery The patients sat upright.

No	t_0	m	σ	P	$(m\sigma^2)^{-\frac{1}{2}}$	Q_{sl}	Q_{rw}
1a	13.18	8.34	0.529	66	0.654	4.24	4.45
1b	10.58	10.49	0.388	66	0.796	3.81	4.23
2a	10.19	10.70	0.378	72	0.810	5.78	6.36
2b	12.19	10.21	0.439	78	0.714	5.60	5.90
3a	9.89	12.15	0.399	96	0.718	4.36	4.36
3b	8.69	12.39	0.375	99	0.757	4.13	4.21
4a	10.03	11.66	0.357	81	0.821	3.92	3.99
5a	18.5?	10.28	0.301	83	0.709	5.72	5.95
5b	16.00	11.70	0.312	87	0.918	5.35	5.69

Groningen curves

8a and 8b injections in the right left pulmonary artery respectively 5b in the vena cava superior all others in the stem of pulmonary artery Measurements in the brachial artery no 1 left, all the others right. The patients lay flat.

No	t_0	m	σ	P	$(m\sigma^2)^{-\frac{1}{2}}$	Q_{sl}	Q_{rw}
1a	7.07	8.38	0.592	51	0.584	3.7	3.8
1b	6.40	10.74	0.438	50	0.696	4.1	4.2
2	0.51	10.48	0.526	78	0.587	2.9	3.3
3a	4.52	4.78	0.352	85	1.298	7.4	7.4
3b	3.97	4.86	0.329	75	1.377	7.9	8.4
4a	2.85	6.21	0.267	71	1.504	8.7	9.2
4b	3.73	5.76	0.271	69	1.538	8.5	9.0
4c	2.93	6.62	0.257	66	1.514	8.6	9.1
5a	0.63	6.20	0.252	73	1.591	9.5	10.0
5b	1.53	6.51	0.264	75	1.482	9.8	9.8
6a	6.05	4.98	0.516	82	0.869	7.6	8.2
6b	4.30	5.89	0.565	90	0.730	6.1	6.3
6c	4.32	4.19	0.426	121	1.146	6.8	7.3
7a	5.72	5.20	0.440	111	0.991	7.0	7.1
7b	6.59	4.79	0.533	96	0.858	7.0	7.1
7c	6.32	5.00	0.480	114	0.931	7.8	8.2
8a	4.12	7.00	0.293	63	1.274	6.0	7.3
8b	3.78	3.88	0.398	84	1.038	6.0	7.7
8c	4.83	5.68	0.404	86	1.288	6.0	6.6

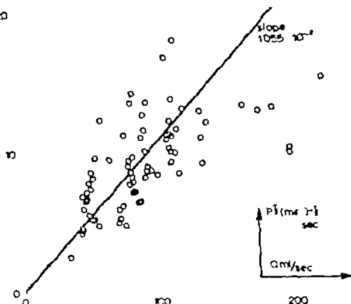


Fig. 5.

As Fig. 4, showing the same line through the origin.

Curves obtained at the cardiovascular research unit, Hammersmith, London, by Dr L T Gabe, Dr J P Shillingford and Dr M Thomas. The patients lay flat with their feet sloping 20° downwards. Injections in the vena cava superior close to the right atrium, measurements in the lobe of an ear

The patients raised their feet after 2 or 3 curves had been made; only those made before the change in position are included, 75 curves from 32 sets.

log ordinate-log abscissa, which allows for increasing scatter further from the origin – is almost the same as the log of the slope in Fig. 4 The correlation was slightly better – and significantly so – with $P^{\frac{1}{2}}$ included than without

In interpreting curves obtained during or after exercise, or following a change in posture when lying down, or when breathing oxygen additional factors are involved. All these analyses will be discussed in another paper

The results from patients at rest provide a strong *prima facie* case supporting a random walk interpretation of dye dilution curves. In the remainder of the paper we shall consider some surprising consequences of this.

TABLE 5

Lognormal parameters for dye-dilution curves from Penarth and Groningen as in Fig 4

t_0 (sec) lower bound

m (sec) σ mean and standard deviation of $\log_e(t-t_0)$

P (min $^{-1}$) pulse frequency

Q_{sl} Q_{rw} (l/min) cardiac outputs, semilog and random walk extrapolations.

The numbers correspond to different patients curves with the same numbers were obtained at one sitting

Penarth curves

Injections in the left brachial vein about 20 cm proximal to the elbow measurements in the left brachial artery The patients sat upright.

No.	t_0	m	σ	P	$(m\sigma^2)^{-1}$	Q_{sl}	Q_{rw}
1a	13 18	8.34	0.5 0	60	0 054	4.24	4.45
1b	10.58	10 49	0 388	60	0 796	3.81	[illegible]
[illegible]a	10 10	10 70	0.378	7[illegible]	0 810	5.78	6.36
[illegible]b	12 10	10.21	0 439	78	0 714	5 60	5 90
3a	9.80	12 15	0.390	90	0 718	4.36	4.36
3b	8 69	12 39	0.375	90	0 757	4 13	4.21
4a	10 03	11 60	0.357	81	0 821	3 9[illegible]	3 99
5a	18.5	10 28	0 391	83	0 799	5 72	5.95
5b	10 00	11 70	0.312	87	0 018	5 35	5.65

Groningen curves

8a and 8b injections in the right left pulmonary artery respectively 5b in the vena cava superior all others in the stem of pulmonary artery Measurements in the brachial artery no 1 left, all the others right The patients lay flat.

No	t_0	m	σ	P	$(m\sigma^2)^{-1}$	Q_{sl}	Q_{rw}
1a	7 07	8.38	0.592	51	0.584	3 7	3.8
1b	6 40	10 74	0 438	50	0 606	4 1	4.2
2	6 51	10 48	0.526	78	0.587	2.9	3.3
3a	4 52	4 78	0.35	85	1.298	7 4	7 4
3b	3 07	4 86	0.329	75	1 377	7 9	8.4
4a	2 65	0.21	0.207	71	1.504	8.7	9.2
4b	3 73	5 76	0 [illegible]71	69	1.538	8.5	9 0
4c	2.93	6 62	0.257	66	1.514	8 6	9 1
5a	0 63	6.20	0 252	73	1.591	0 5	10 0
5b	1.53	6.51	0.204	75	1 482	9 8	9.8
6a	0 05	4 98	0.516	82	0 800	7 6	8.2
6b	4.30	5 89	0.565	90	0 730	6.1	6.3
6c	4.32	4 19	0 426	121	1 146	6 8	7.2
7a	5 72	5.26	0 440	111	0 991	7 0	7 1
7b	6.59	4 79	0.533	90	0 858	7 0	7 1
7c	6.3	5 00	0 480	114	0 931	7.8	8.[illegible]
8a	4 12	7 00	0.293	83	1.274	6 0	7.3
8b	3 78	3 88	0.398	84	1 038	0 0	7 7
8c	4.83	5 68	0 404	86	1.288	0 0	0 0

If we consider only the first arrivals (as considered by Sheppard) we arrive at a distribution simply related to (7.1) the number first arriving during the same time interval t to $t+dt$, which we write $y_{1a}(t)\,dt$ is in fact given by

$$y_{1a}(t) = 2y_1(t)\mu/(\mu+t). \tag{7.2}$$

If the rate of spreading out is small so that t is seldom very different from μ, these two distributions are nearly the same. This agrees with common sense in that case few particles change places. But in general the two distributions will be considerably different.

The local density distribution too is different from (7.1). For a flow f across an area of cross section A_a, the concentration at the point of measurement—i.e. in a small interval of length at this point in the direction of flow is (see Appendix A) equal to $y_c f/A$ (cf. Eq. 4.1) and we have

$$y_1 = \tfrac{1}{2}\left(\frac{\mu}{t}+1\right)y_c. \tag{7.3}$$

So the net rate of movement to the right is *not* equal to the local concentration times the volume flow fA_a except at the instant when the median particle of tracer is at the place of measurement.

The formula for the cardiac output fortunately remains correct, provided the dye-dilution curve is in fact the record of the local concentration My_c/fA_a as a function of time (M is the mass injected). The cardiac output Q equals by definition the volume flow fA_a, and it is easy to show (see Appendix A and C) that y_c is a probability function of t so that it follows that the observed area of the complete curve

$$A = \int_0^\infty \frac{My_c}{Q}\,dt = \frac{M}{Q} \tag{7.4}$$

We shall now reexamine the detailed derivation of the formula $Q\bar{t}$ for the central blood volume.

8. THE CENTRAL BLOOD VOLUME IN THE RANDOM WALK MODEL

There is a difficulty in defining this volume if the sites of both injection and measurement are peripheral. In the random walk model this must be done in terms of the median particle that is

7 WHAT IS MEANT BY A DISTRIBUTION OF PASSAGE TIMES?

Most *surprisingly* it proves difficult to answer this question We can write down the proportion of injected particles that are beyond the place of measurement at time t and deduce its rate of increase With the notation of the previous section this comes to

$$y_f(t) = e^{\lambda}\left(\frac{\lambda}{2\pi}\right)^{\frac{1}{2}} \frac{1}{2t}\left\{\left(\frac{\mu}{t}\right)^{\frac{1}{2}} + \left(\frac{t}{\mu}\right)^{\frac{1}{2}}\right\} \exp -\tfrac{1}{2}\lambda\left(\frac{t}{\mu} + \frac{\mu}{t}\right) \quad (7\ 1)$$

(this is proved in Appendix A)

In other words (for a unit number or mass of injected particles) *there are* $y_f(t)\,dt$ *more particles beyond the place of measurement* – (at distance μf $\lambda = \mu f/L$) at time $t + dt$ than at time t But this is not the number of particles with passage times between t and $t + dt$ It is the difference between the number of particles crossing from left to right and the number crossing from right to left. If we consider that the particles have no individuality and we rank them from left to right we can define a passage time distribution for percentiles (i.e the time taken by e g the $\frac{1}{2}M$th particle in ranking order if M particles are injected irrespective of which *particle this is) Then every particle will be counted once* and the probability distribution of the passage times of the percentiles is given by (7 1)

In a completely different situation the related idea of percentile pairing was introduced This was a longitudinal study of a group of coal miners (Wise and Oldham, 1963) Some of the respired dust was retained *in their* lungs and this gradually produced changes on their chest X rays. The amount *of this X ray abnormality (simple pneumoconiosis) could only be categorised* into coarse groups, whilst it was known to vary continuously The frequency distribution of these coarse groups was known for the same miners at two points in time separated by 3 years. The increase for each percentile for the two continuous distributions of abnormality was calculated. This yielded much more information than that of the change for each individual miner The change between the two distributions – by percentile pairing – is itself a complete distribution which would be exactly correct *if every* individual stayed in the same position in ranked order of abnormality *His actual amount of abnormality generally increasing, is analogous* to the distance moved by a particular injected particle in the blood stream.

We shall examine the system with one inlet and one outlet but many different pathways between them. We consider the particles that are in the system at time $t = 0$. Then we consider all the particles that leave the system at times between t' and $t' + dt'$. These particles are then classified according to the time they took to get through the system which we shall call t_p. Then $C(t_p)\,dt_p/A$ of them took times between t_p and $t_p + dt_p$, where $C(t)$ is assumed to be the observed dye-dilution curve and its (total) area is A. If this time t_p was greater than t' they were in the system at $t = 0$; if it was less they were not then in the system.

The next step is to write down the rate at which these particles leave the system. If Q is the rate of (volume) flow this is

$$QC(t_p)\,dt_p/A \tag{8.2}$$

so the total volume of the particles having these transit times, that were in the system at time $t = 0$ is

$$V = \int_0^{t_p} \left\{ \frac{QC(t_p)}{A} dt_p \right\} dt' = t_p \frac{QC(t_p)}{A} dt_p \tag{8.3}$$

So the total volume is obtained by integrating this over all values of t_p, which shows that this equals $Q\bar{t}_p$. This is the accepted proof.

In the random walk model, the question at once arises—which passage time do we take? The proportion $C(t_p)\,dt_p/A$ must include particles that have arrived at the exit of the system for the second time or more. But if the time taken to get through the system to the exit for the first time t_{1s}, is less than the time t' at which they leave the system they would have been outside it—to the left (with the convention of flow from left to right as in Fig. 3) of the place of injection—at $t = 0$ yet t_p could still be greater than t'. If the first passage time t_{1s} is greater than t_p they were certainly within the system at $t = 0$.

If the first passage time is the criterion, every particle in the defined volume is counted once and only *once*. The expression for the volume flow becomes $Q y_{1s}(t_p)\,dt_p$. From the definition of flow this is still correct as a mean rate of leaving the system; we are no longer dealing with a selected population of particles that were at the entrance at $t = 0$ as in (7.3) but with a certain proportion of all the particles that are at the exit at time t'.

injected We can consider the time t_{ra} that this particle takes to reach the right atrium Then we have to consider all other sites in the blood with the same arrival times t_{ra} The central volume must include the volume of blood between all these sites and the right atrium It also includes of course the volume of the right and the left heart and the lung capillaries about which there is no difficulty On leaving the left ventricle this median particle takes a further time t_{vm} to reach the site of measurement, and the central volume must include all other places in the circulation which can be reached by median particles in time t_{vm} or less from leaving the left ventricle

There is no difficulty if injection is just outside the right atrium and the measurements are in the ascending aorta But in fact it is generally agreed that the central volume must be defined in this way For the random walk model the concept of a median particle is essential We recall that it changes its identity always such that half the particles moving along the same route have travelled further (from the left heart or will travel further to reach the right heart as the case may be) and the other half have travelled less far

The results for uniform flow along a tube of constant cross section are consistent with this. The mean rate Q at which particles cross a surface of area A_0 at right angles to the direction of flow is certainly equal to fA_0 and the volume between the injection and measurement sites V is exactly

$$V = A_0\bar{x} = fA_0\mu = Q\mu \tag{8.1}$$

It is equally clear that this is not equal to $Q\bar{t}$ where $\bar{t}$ is the statistical mean of the dye-dilution curve regarded as a probability distribution This mean $\bar{t}$ (Appendix C) equals $\mu\,(1 + 1/\lambda)$ for local random walk curves, for typical values of λ it is therefore between 5 % and 25 % greater than the passage time of the median particle along such a tube

The general proof by MEIER and ZIERLER (1954) (see also ZIERLER, 1962) that the central blood volume equals $Q\bar{t}$ is certainly correct on the assumptions they have given Yet this proof must apply to flow in a uniform tube as well as to the complex system in the human body So we shall reexamine this proof and consider which basic assumptions break down for the random walk model.

central sites of injection and measurement, the curves are probably more skew than most of those we have examined an average value of σ round about 0·4 (or $\lambda = 6$) is quite reasonable. A 12 % excess in the blood volume assumed to be equal to $Qt̄$ could easily be about the same as the ratio of the two expressions for the central blood volume.

APPENDIX A

THE TWO RANDOM WALK DISTRIBUTIONS WITH NO RESTRICTION TO FIRST PASSAGES

Suppose that a unit mass of particles is injected at the origin. The particles are carried with a stream and move (say) to the right. At time μ after injection the median particle has travelled a distance $f\mu$.

We consider what is happening at this point. At time t the median particle is at ft and the other particles are distributed normally around this distance with variance kft.

Then the proportion of particles that have passed the fixed point at time t is given by

$$P(x > f\mu) = \frac{1}{\sqrt{2\pi kft}} \int_{f\mu}^{\infty} \exp - \frac{x^2}{2kft} \, dx \qquad (A.1)$$

Putting $x/(kft)^{\frac{1}{2}} = X$

$$P = \frac{1}{\sqrt{2\pi}} \int_{\frac{f(\mu - t)}{\sqrt{kft}}}^{\infty} \exp - \tfrac{1}{2} X^2 \, dX \qquad (A.2)$$

From this we can deduce the *net* rate at which particles pass the fixed point. Most particles at each *instant* are getting further from the point of injection, but some will be returning. This net rate is given by

$$\frac{\partial P}{\partial t} = \tfrac{1}{2}\sqrt{\frac{f}{2\pi k}} \left(\mu t^{-\frac{3}{2}} + t^{-\frac{1}{2}}\right) \exp - \frac{f(t-\mu)^2}{2kt} \qquad (A.3)$$

Putting $\lambda = f\mu/k$ yields Eq. (7.1).

The local concentration in this one-dimensional model must be a linear density. So we require the proportion of particles, at this instant of time at distances between $x = f\mu$ and $x = f\mu + dx$.

It follows that the volume of particles in the system at $t=0$ with first passage times between t_p and t_p+dt_p is

$$\int_{t'=0}^{t_p} \{Qy_{1s}(t_p)\, dt_p\}\, dt' = Qt_p y_{1s}(t_p)\, dt_p \tag{8.4}$$

Integrating this over all possible first passage times gives the volume of the system

$$V = Q\mu \tag{8.5}$$

since the mean of the first passage distribution is μ (Appendix C).

This seems to follow equally if we have to regard the spreading out as beginning at a later instant than the moment of injection. In other words, the above arguments are equally valid for a median transit time equal to $\mu+t_0$ as discussed in section 5.

The conclusion is then that the central blood volume is overestimated: the mean of the local random walk curve (as for the uniform tube) is

$$\bar{t}_c = \mu\left(1+\frac{1}{\lambda}\right) \tag{8.6}$$

so that the usual formula overestimates the volume $Q\mu$ by an amount $Q\mu/\lambda$.

For a typical value of the lognormal standard deviation of 0.4, $\lambda=6$; then the difference would be about 15 %. The random walk parameter μ corresponds approximately to the lognormal m; as we have seen, the percentage difference between $t_0+\mu$ and $t_0+\bar{t}_c$ would be about 10 %. The actual difference obtained from lognormal approximations, varies between 5 % and 10 % in most cases.

SCHLANT *et al.* (1959) have made a direct comparison. They determined the actual volume of blood in the heart and the lungs by labelling red cells with ^{51}Cr. The volume calculated from the dye-dilution curves in the usual way, on the average for 22 dogs, was 1.12 times that of the actual blood volumes. KORNER (1962) suggests that this was because there would be a lower *haematocrit ratio* in the smaller pulmonary blood vessels. Also, the mean passage times obtained from semi-log extrapolations may not be quite reliable. But a simpler explanation is the use of the usual formula for the blood volume. Injections were just at the entrance to the right atrium and measurements in the ascending aorta. With these

central sites of injection and measurement, the curves are probably more skew than most of those we have examined an average value of σ round about 0·4 (or $\lambda = 6$) is quite reasonable. A 12 % excess in the blood volume assumed to be equal to $Q\bar{t}$ could easily be about the same as the ratio of the two expressions for the central blood volume

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Putting $\lambda = f\mu/k$ yields Eq (7 1).

The local concentration in this one-dimensional model, must be a linear density So we require the proportion of particles, at this instant of time at distances between $x = f\mu$ and $x = f\mu + \mathrm{d}x$

(Since the running variable has been changed to X we can use x for a particular distance without confusion) Restoring x for $l\mu$ in (A 2) we have

$$\frac{\partial P}{\partial x} = -\frac{1}{\sqrt{2\pi\lambda t}}\exp\left\{-\frac{(x-\mu t)^2}{2\lambda t}\right\} \qquad \text{(A.4)}$$

So the local (linear) concentration at distance μl that is the number of particles per unit distance in a small region round this place of measurement is per unit mass injected equal to $-\partial P/\partial x$ at $x = \mu l$. Again putting $\lambda = (l\mu)/L$ this reduces to

$$Y_c = \frac{1}{\mu l}e^{\lambda}\left(\frac{\lambda}{2\pi}\right)^{\frac{1}{2}}\left(\frac{\mu}{t}\right)^{\frac{1}{2}}\exp\left\{-\tfrac{1}{2}\lambda\left(\frac{t}{\mu}+\frac{\mu}{t}\right)\right\} \qquad \text{(A.5)}$$

APPENDIX B

THE MAXIMUM AND THE POINTS OF INFLECTION OF THE LOCAL RANDOM WALK FUNCTION

The probability function is

$$y = \frac{e^{\lambda}}{\mu}\left(\frac{\lambda}{2\pi}\right)^{\frac{1}{2}}\left(\frac{\mu}{t}\right)^{\frac{1}{2}}\exp\left\{-\tfrac{1}{2}\lambda\left(\frac{t}{\mu}+\frac{\mu}{t}\right)\right\} \qquad \text{(B 1)}$$

If we put $t/\mu = \tau = e^{u}$, $a = e^{\lambda}(\lambda/2\pi)^{\frac{1}{2}}$

$$y = \frac{a}{\mu}e^{-\frac{1}{2}u-\lambda\cosh u} \qquad \text{(B 2)}$$

Hence $dy/d\tau = -y/\tau\,(\tfrac{1}{2}+\lambda\sinh u)$ (B 3)

If the mode is $\tau_M = \exp u_M$ then

$$\sinh u_M = -1/(2\lambda) \qquad \text{(B 4)}$$

Further

$$d^2y/d\tau^2 = y/\tau^2\,\{(\tfrac{1}{2}+\lambda\sinh u)^2+\tfrac{1}{2}+\lambda\sinh u-\lambda\cosh u\} \qquad \text{(B 5)}$$

Putting

$$\tfrac{1}{2}+\lambda\sinh u = U \qquad \text{(B 6)}$$

$\lambda^2\cosh^2 u = (U-\tfrac{1}{2})^2+\lambda^2$ and the equation for the points of inflection reduces to

$$U^4+2U^3+U = \lambda^2+\tfrac{1}{4} \qquad \text{(B 7)}$$

This was solved for particular values of λ by inverse interpolation.

APPENDIX C

THE MOMENTS AND CUMULANTS OF THE THREE RANDOM WALK FUNCTIONS

We shall put $\tau = t/\mu$ and find the moments and cumulants of τ the sth moment of t is clearly μ times the sth moment of τ The probability functions are multiplied by $\exp \tau Z$ and integrated from $\tau = 0$ to ∞ the coefficient of $Z^s/s!$ is the sth moment The three moment generating functions are (writing $a = \sigma^{2} (\lambda/2\pi)^{\frac{1}{2}}$
First passage

$$M_{1s}(Z) = a \int_0^\infty \tau^{-3/2} \exp\{-\tfrac{1}{2}\lambda(\tau + 1/\tau) + \tau Z\}\, d\tau \qquad (C\,1)$$

Net rate of flow

$$M_f(Z) = a \int_0^\infty \tfrac{1}{2}(\tau^{-3/2} + \tau^{-1/2}) \exp\left\{-\tfrac{1}{2}\lambda\left(\tau + \frac{1}{\tau}\right) + \tau Z\right\} d\tau \qquad (C.2)$$

Local density

$$M(Z) = a \int_0^\infty \tau^{-1/2} \exp\left\{-\tfrac{1}{2}\lambda\left(\tau + \frac{1}{\tau}\right) + \tau Z\right\} d\tau \qquad (C.3)$$

It follows that $M(Z) = dM_{1s}(Z)/dZ$ (C.4)

and $M_f(Z) = \tfrac{1}{2}\{M_{1s}(Z) + dM_{1s}(Z)/dZ\}$ (C.5)

To evaluate $M_{1s}(Z)$ we put $\tau = u^{-2}$ and obtain

$$M_{1s}(Z) = 2a \int_{u=0}^{\infty} \exp\left(\frac{Z - \tfrac{1}{2}\lambda}{u^2} - \tfrac{1}{2}\lambda u^2\right) du. \qquad (C.6)$$

Putting $1 - 2Z/\lambda = \eta^2$ this reduces to

$$2a \exp - \lambda\eta \int_0^\infty \exp - \tfrac{1}{2}\lambda (u - \eta/u)^2\, du. \qquad (C.7)$$

Now putting $u - \eta/u = v\lambda^{-\frac{1}{2}}$ $2u = v\lambda^{-\frac{1}{2}} + (4\eta + v^2/\lambda)^{\frac{1}{2}}$
and we find that

$$M_{1s}(Z) = 2a \exp(-\lambda\eta) \int_{-\infty}^{+\infty} (\exp - \tfrac{1}{2}v^2) \left\{\frac{1}{\sqrt{\lambda}} + \frac{v}{2\lambda}\left(4\eta + \frac{v^2}{\lambda}\right)^{-\frac{1}{2}}\right\} dv \qquad (C.8)$$

For the odd function on the right the integral vanishes, leaving

$$M_{1a}(Z) = a\exp(-\lambda\eta)\sqrt{\frac{2\pi}{\lambda}} = e^{\lambda(1-\varphi)} \qquad (C\ 9)$$

So the cumulant generating function

$$\log M_{1a}(Z) = \lambda\left\{1-\left(1-\frac{2Z}{\lambda}\right)^{\frac{1}{2}}\right\} \qquad (C\ 10)$$

$$= Z + \frac{1}{\lambda}\frac{Z^2}{2!} + \frac{1\ 3}{\lambda^2}\frac{Z^3}{3!} + \frac{1\ 3\ 5}{\lambda^3}\frac{Z^4}{4!} + \frac{1\ 3\ 5\ 7}{\lambda^4}\frac{Z^5}{5!} \qquad (C\ 11)$$

From (C 10) (C 4) and (C 5) we find that

$$\log M_c(Z) = -\tfrac{1}{2}\log\left(1-\frac{2Z}{\lambda}\right)+\lambda-\lambda\left(1-\frac{2Z}{\lambda}\right)^{\frac{1}{2}} \qquad (C\ 12)$$

$$= Z\left(1+\frac{1}{\lambda}\right)+\frac{Z^2}{2!}\left(\frac{1}{\lambda}+\frac{2}{\lambda^2}\right)+\frac{Z^3}{3!}\left(\frac{1\ 3}{\lambda^2}+\frac{2\ 4}{\lambda^3}\right)+$$

$$+\frac{Z^4}{4!}\left(\frac{1\ 3\ 5}{\lambda^3}+\frac{2\ 4\ 6}{\lambda^4}\right)+\frac{Z^5}{5!}\left(\frac{1\ 3\ 5\ 7}{\lambda^4}+\frac{2\ 4\ 6\ 8}{\lambda^5}\right)+ \qquad (C\ 13)$$

$$\log M_t(Z) = \lambda-\lambda\left(1-\frac{2Z}{\lambda}\right)^{\frac{1}{2}}-\log 2+\log\left\{1+\left(1-\frac{2Z}{\lambda}\right)^{-\frac{1}{2}}\right\} \qquad (C\ 14)$$

$$= Z\left(1+\frac{1}{2\lambda}\right)+\frac{Z^2}{2!}\left\{\frac{1}{\lambda}+\frac{1}{\lambda^2}\left(2-\frac{1\ 3}{4}\right)\right\}$$

$$+\frac{Z^3}{3!}\left\{\frac{1\ 3}{\lambda^2}+\frac{1}{\lambda^3}\left(2\ 4-\frac{1\ 3\ 5}{6}\right)\right\}$$

$$+\frac{Z^4}{4!}\left\{\frac{1\ 3\ 5}{\lambda^3}+\frac{1}{\lambda^4}\left(2\ 4\ 6-\frac{1\ 3\ 5\ 7}{8}\right)\right\}$$

$$+\frac{Z^5}{5!}\left\{\frac{1\ 3\ 5\ 7}{\lambda^4}+\frac{1}{\lambda^5}\left(2\ 4\ 6\ 8-\frac{1\ 3\ 5\ 7\ 9}{10}\right)\right\} \qquad (C\ 15)$$

The cumulants are the coefficients of $Z^n/n!$ in these expansions. In the general case with time parameter μ the nth cumulant is μ^n times this coefficient

All this work developed from analyses of actual tracings. I would like to thank Professor W G Zijlstra Dr F ten Hoor Dr J E. Cotes, and Dr J P Shillingford for providing them, B Th. M van Oosten, who helped with analysing them and Professor J G Defares for many stimulating discussions.

SUMMARY

The fits to tracer dilution curves by some mathematical probability curves are discussed. These fits are based on the triangle formed by the two tangents at the points of inflection on the rising and falling limbs. Lognormal, random walk and gamma curves with the same inflection triangle are compared, and lognormal and random walk curves for a whole range from nearly symmetrical curves (and triangles) to very skew ones. Tables for numerical analyses of observed curves in terms of random walk ones are given these include the height ratios (of triangle to maximum of curve) and area ratios (of triangle to area under the complete curve) needed for estimating cardiac outputs.

The known model is discussed for a Gaussian distribution of distances of injected particles along linear stream, spreading out as they move with the stream. It is shown that this leads to tw different random walk distributions. The dye-dilution curves probably correspond t the one for "local density Its parameters are interpreted in terms of those of the model. This interpretation agrees qualitatively with observed variations in asymmetry of tracer dilution curves for mammals if the time rat of spreading out, for injected tracer moving past the place of measurement, is roughly constant The results of analysing curves from patients at rest with widely varying cardiac outputs are shown to agree with this.

The concept of a distribution of passages times has to be redefined for random walk model, when individual particles are changing places with one another The central blood volume is redefined and derived in terms of the curve; the usual formula is found to be invalid for this model.

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RATIONALIZATION AND OPTIMALIZATION OF HAEMODIALYSIS PROCEDURE

BY

J. DE BOER [1], J. ROUKENS [2], J. W. DUYFF AND M. MANDEL [3]

1 INTRODUCTION

In haemodialysis the problem is one of restoring and maintaining acceptable concentrations of several plasma constituents rather than that of removing one end product of metabolism. Ideally at the end of a session all key substances involved should have plasma concentrations lying in a range between preset maximum and minimum levels. This poses some problems in view of the fact that specific permeation rates as well as initial (percentual) concentration excesses (or deficits) and production rates differ for

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the substances involved As far as could be ascertained no quantitative treatment of the subject has so far been given. The present is an attempt at such a treatment intended to furnish a basis for optimalization of the procedure For the sake of simplicity removal of substances alone will be considered the treatment given however can also be applied to the restoration of normal plasma levels in the case of a concentration deficit.

Of the many variables involved two cannot be voluntarily changed vi the distribution volume of any given substance in the body (its space) and the rate at which end products of metabolism to be removed are produced Those which can be changed include the nature and surface area of the membrane, the volume and the composition of the fluid in the artificial kidney and the duration and frequency of dialysis sessions

For any given substance involved the problem is the following the substance in question is present in the extra-cellular space at a given initial molar plasma concentration Plasma concentration at the end of a session is to lie within a range of permissible concentrations the lower end of this range may or may not be zero depending on the substance in question For a given permeation rate (depending on nature and thickness of the dividing membrane) membrane surface area and counter fluid volume and for a given production rate and initial concentration in the outside fluid what will the plasma concentration be at any given time from the beginning of dialysis?

What is the influence of membrane surface area and of outside fluid volume as parametric values on dialyzer performance?

2. THEORY

Consider a two-compartment system as in Fig 1 The two compartments of volume V_1 and V_2 respectively contain a common solvent which passes freely through the membrane of thickness Δx and of surface area S separating them A solute which is able to pass through the membrane with a specific permeation rate D is present in V_1 in molar concentration C_1 and in V_2 in molar concentration C_2 $\Delta C = (C_1 - C_2)$ being positive. D is assumed to be a constant independent of the concentration both of the permeating solute and of any other substances present in

either compartment. At time zero $C_{1_0} > C_{2_0} > 0$. Solute is added to the solution in V_1 at a constant rate of ϱ particles per time unit. Hydrostatic forces are assumed to be compensated for and provisions for thorough mixing in both compartments are supposed to have been made.

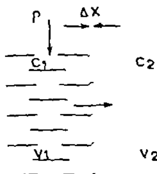


Fig. 1.

V stands for the extracellular fluid compartment, V_2 for dialyser fluid volume. Plasma concentration is supposed to be representative of concentration in the extracellular fluid, so Donnan effects are left out of consideration ϱ is supposed to be independent of momentary plasma concentration.

Now $\frac{d\Delta C}{dt} = \frac{dC_1}{dt} - \frac{dC_2}{dt} = \frac{1}{V_1} \; \frac{dn_1}{dt} - \frac{1}{V_2} \; \frac{dn_2}{dt}$ where n is the number of moles of solute

$\frac{dn_1}{dt} = \varrho - \frac{D\,S}{dx} \; \Delta C = \varrho - D' \; \Delta C$ where D' is the membrane constant.

$\frac{dn_2}{dt} = D' \; \Delta C$

So $\frac{d\Delta C}{dt} = \frac{1}{V_1}(\varrho - D' \; \Delta C) - \frac{1}{V_2} \; D' \Delta C = \frac{\varrho}{V_1} - D' \left(\frac{V_1 + V_2}{V_1 V_2}\right) \Delta C$

For $\frac{\varrho}{V_1} = A$ and $D' \; \left(\frac{V_1 + V_2}{V_1 V_2}\right) = B$ that is for $\frac{A}{B} = \frac{\varrho}{D'} \left(\frac{V_2}{V_1 + V_2}\right)$

we have $\frac{d\Delta C}{dt} = A - B \; \Delta C$

Integration yields $\Delta C = K e^{-Bt} + A/B$ where K is the integration constant as found from boundary conditions for $t = 0$

$$\Delta C_0 = K + A/B \quad \text{or} \quad K = \Delta C_0 - A/B \quad \text{so}$$

$$(C_1 - C_2) = \Delta C_t = (\Delta C_0 - A/B)e^{-Bt} + A/B \tag{1}$$

Now since overall mass balance is given by

$$n_1 + n_2 = n_{1_0} + n_{2_0} + \rho t$$

where n_{1_0} and n_{2_0} stand for the number of moles of solute at $t = 0$ in the respective compartments

$$C_1 \frac{V_1}{V_2} + C_2 = C_{1_0} \frac{V_1}{V_2} + C_{2_0} + \frac{\rho t}{V_2} \tag{2}$$

Addition of (1) and (2) yields

$$C_1 \frac{V_1 + V_2}{V_2} = C_{1_0} \frac{V_1}{V_2} + C_{2_0} + \frac{\rho t}{V_2} + \left(\Delta C_0 - \frac{A}{B}\right) e^{-Bt} + \frac{A}{B} \quad \text{from which}$$

$$C_1 = \frac{C_{1_0} V_1 + C_{2_0} V_2 + \rho t + V_2\left[\left(\Delta C_0 - \frac{A}{B}\right) e^{-Bt} + \frac{A}{B}\right]}{V_1 + V_2} \tag{3}$$

$$C_2 = \frac{C_{1_0} V_1 + C_{2_0} V_2 + \rho t - V_1\left[\left(\Delta C_0 - \frac{A}{B}\right) e^{-Bt} + \frac{A}{B}\right]}{V_1 + V_2} \tag{3'}$$

It will be seen that all variables involved enter in the Eqs. (3) in a complicated way and numerical calculation will be necessary to arrive at a solution in any given case. More specifically, though the efficiency of a dialyzer increases with membrane surface area and with outside fluid volume and decreases with increase of membrane thickness, the relations are far from being linear and are not easily surveyable.

For some special cases the equations reduce to simpler forms.

(a) $C_{2_0} = 0$ i.e. initial concentration in the outside fluid is zero

$$C_1 = \frac{C_{1_0} V_1 + \rho t + V_2\left[\left(C_{1_0} - \frac{A}{B}\right) e^{-Bt} + \frac{A}{B}\right]}{V_1 + V_2} \tag{3a}$$

$$C_1 = \frac{C_{1_0}V_1 + \rho t - V_1\left[\left(C_{1_0} - \frac{A}{B}\right) e^{-Bt} + \frac{A}{B}\right]}{V_1 + V_2} \qquad (3\ a)$$

(b) $\rho = 0$ i.e. there is no further production of the substance to be removed

$$C_1 = \frac{C_{1_0}V_1 + C_{2_0}V_2 + V_2\, \Delta C_0\, e^{-Bt}}{V_1 + V_2} \qquad (3b)$$

$$C_2 = \frac{C_{1_0}V_1 + C_{2_0}V_2 - V_1\, \Delta C_0\, e^{-Bt}}{V_1 + V_2} \qquad (3'b)$$

(c) $\rho = 0$ $C_{2_0} = 0$ as in the case where an exogenous toxic substance is to be removed

$$C_1 = \frac{C_{1_0}V_1 + C_{1_0}V_2\, e^{-Bt}}{V_1 + V_2} = C_{1_0}\frac{V_1 + V_2\, e^{-Bt}}{V_1 + V_2} \qquad (3c)$$

$$C_2 = \frac{C_{1_0}V_1 - C_{1_0}V_1\, e^{-Bt}}{V_1 + V_2} = C_{1_0}\frac{V_1(1 - e^{-Bt})}{V_1 + V_2} \qquad (3\ c)$$

If we assume the permeation rate of one of the substances involved to be independent of the concentration of the others, a two-substance problem can be stated generally as follows

There are two substances, each with its own equivalent distribution space, its own initial percentual upward deviation from the normal plasma level, its own rate of production and its own specific permeation rate

In general, the plasma concentration of both will not be back to normal at the same time, and bringing back one to the desired level will entail the danger of depletion of the other unless special precautions in the sense of providing for an initial non zero concentration in the outside fluid, are taken. Given upper and lower permissible concentration levels of both substances what should this initial concentration be and how long should the session last? How long after the kidney has been detached should a second dialyzing period be started if, for each substance, a maximum plasma level has been set which must not be exceeded, how long should this session (and succeeding "maintenance" sessions) last, and what is to be the interval between successive maintenance sessions?

1.00

concentration/time curves for creatinin
membrane area is 2.0 square meters
artif kidney volumes given in liters

extracell. conc. (grams/liter x10⁻¹)

time (hrs)

50

0.50

100

150

200

700

0 0 12.00 24.00

Fig. 2.

1.00

concentration/time curves for creatinin
membrane area is 2.0 square meters
artif kidney volumes given in liters

extracell. conc. (grams/liter x10⁻¹)

time (hrs)

0.50

50

100

150

200

250

400

700

0 0 12.00 24.00

Fig. 3.

1.00

concentration/time curves for creatinn
membrane area is 1.5 square meters
artif kidney volumes given in liters

extracell.conc.
(grams/liter x10⁻¹)

time (hrs)

0.50

50
100
150
200
250
300
400
700

0 0
12.00
24.00

Fig. 4.

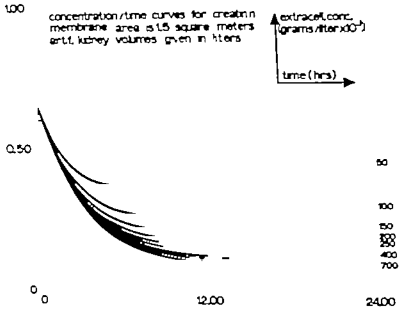


Fig. 5.

To solve problems of this kind Eq (3) was programmed on an IBM 7094–II computer [1]) The figures in the next section are copied from plots delivered by the computer

The permeating substances considered in the following are ureum and creatinin It is well realized that in particular elimination of ureum is not of great importance in haemodialysis The combination creatinin ureum was chosen to illustrate the case because with these substances we are on fairly safe ground as regards the values of the distribution volumes production rates, and specific permeation rates The reasoning applies however to any combination of two or more substances

D values were determined in model experiments (see Appendix) V_1 and ρ values are taken from earlier experiments (DE BOER, J Acta Physiol Pharmacol Neerl. 12 345–372 (1963)) Data are given in Table 1

TABLE I

	D (cm^2 sec $^{-1}$)	V_1 (cm^3)	ρ (mg sec^{-1})
Creatinin	0 35 × 10^{-4}	33 × 10^3	0 013
Ureum	0 58 × 10^{-4}	46 × 10^3	0,575

3 COMPUTER RESULTS

3 1 GENERAL CHARACTER OF PLASMA CONCENTRATION/TIME CURVES

The general shape of the plasma concentration vs time curves is shown in Figs 2–5 Plasma concentration decreases at a diminishing rate until a minimum is reached after which it increases quasi linearly For a given membrane surface area the minimum is reached later and is lower when outside fluid volume is greater also the rate of rise after the minimum has been attained is less. For a given outside fluid volume an increase in membrane area

1) The program is available upon request

leads to a lowering of the minimum plasma concentration reached and to an advance in time of the minimum Its influence on the rate of increase after the minimum has been passed is slight.

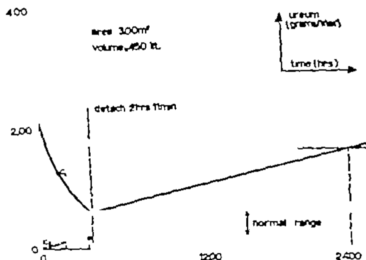


Fig 6.

A specific case is illustrated by Fig 6 The normal range of inside ureum concentrations is arbitrarily put at 20–60 mg/100 ml, the initial concentration at about 216 mg/100 ml and the maximum permissible concentration at 160 mg/ml For the values given the normal range will be reached in 2 hours and 11 min if the kidney is then detached the critical concentration at which it should again be attached will be attained at time 24 h 24 mm, i.e. 22 hours and 13 min after the end of the (first) dialysis session. A session of about 1 h 30 min will then be sufficient to bring the plasma concentration back to the upper limit of the normal range again. If the kidney is then detached the next maintenance session, of equal duration, must start after an interval of 22 h 13 min.

If in the first session, the kidney were not detached at time h 11 min inside (plasma) concentration would diminish further and the minimum would be reached at time 7 h 27 min minimum concentration would be about 29 mg/100 ml.

3 2 A TWO-SUBSTANCE CASE

A two-substance case is illustrated by the plot of Fig 7 which shows concentration *vs time curves for ureum and creatinin.* In the plot the ordinates have been scaled and shifted in such a way that the normal ranges coincide

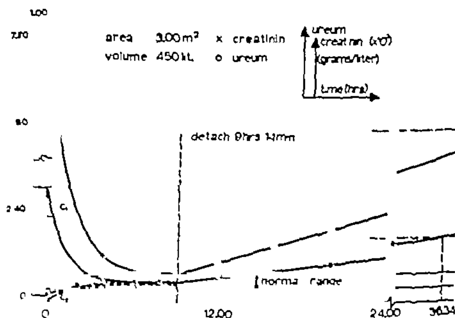


Fig 7

In the plot of Fig 7 that is, for initial plasma concentrations of 300 mg/100 ml for ureum and 10 mg/100 ml for creatinin and for the further numerical values shown the upper limit of the normal range is reached for ureum at time 2 h 57 min.

That for creatinin is reached at 9 h 14 min that is well after the point in time (7 h 52 min) when plasma ureum concentration has passed its minimum concentration of 30 81 mg/100 ml. If at time 9 h 14 min the session is terminated ureum stays within the normal range for another five hours and six minutes Ureum is the first to reach the critical concentration at time 30 h 34 min that is twenty-seven hours and twenty minutes after the end of the first session If the first of the maintenance sessions is started at this moment it will have to last for about 1 h 30 min the interval between maintenance sessions would be 25 h 10 min

If the kidney were not detached at the moment when plasma

creatinin reached the upper limit of the normal range, but when it reached its minimum of about 0 9 mg/100 ml at time 9 h 54 min, ureum would, at the end of the session, be at about 38 mg/100 ml, and its critical concentration would be reached at time 37 h 04 min.

In the example chosen creatinin is the critical substance as far as the duration of dialysis sessions is concerned, because of its far greater initial plasma concentration excess and to a lesser extent because of its lower specific permeation rate ureum, which first reaches the upper permissible plasma concentration because of its high ϱ value, is critical with regard to the interval between dialysis sessions. Depending on numerical values, one substance may or may not be critical in both respects.

In the case of ureum, the minimum permissible plasma concentration is probably zero In those cases, however where a permeating plasma constituent has a non zero minimum permissible plasma concentration, as is the case with plasma electrolytes, initial non zero outside concentrations of one or more of them will, in general, be indicated.

Again for ureum (and creatinin) the production rate is independent of the plasma concentration this is not so for all substances involved. A quantitative treatment of these and similar questions will be given in a separate paper

3.3. Influence of membrane thickness (or material) membrane area, and fluid volume on the performance of a dialyzer

The way in which membrane surface area and outside fluid volume determine dialyzer performance is shown, again for ureum and for creatinin, in Figs. 8 and 9

The–arbitrary–criterium used was whether for a given initial plasma concentration and for a given combination of membrane surface area and kidney fluid volume the substance in question would, in the course of a dialysis session of indeterminate duration, at all reach the upper limit of the normal range For each of the initial plasma concentrations indicated the curves give the minimum area volume combinations sufficient to ensure this. Similar plots can be made if the criterion is the attainment of the high-normal value within a given time

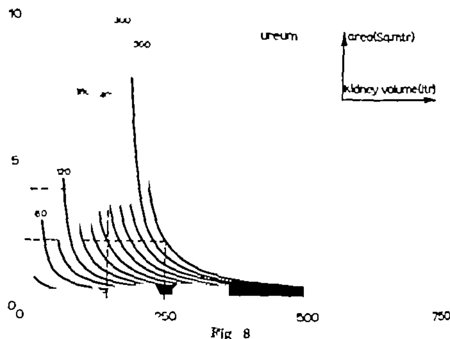


Fig 8

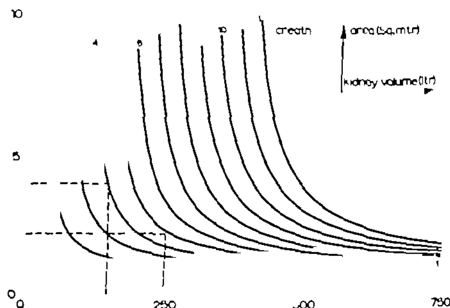


Fig 9

Current haemodialyzors have fluid volumes up to 400 l and membrane areas up to 2 5 m² of course large fluid volumes and large surface areas usually go hand in hand The plots suggest that in attempts at further improvement of performance increase

of membrane surface area would be more promising than increase of fluid volume.

An arbitrarily chosen numerical example illustrates the general picture. A 250 l kidney can get plasma ureum concentration down to high normal from 360 mg/100 ml if membrane area is about 2½ m² A 150 l kidney could not normalize plasma ureum concentration if the initial concentration were over 240 mg/100 ml even for this value, a surface area of about 4 m² would be necessary a 250 l one square meter kidney would perform similarly

The imagined 250 l, 2½ m² kidney could normalize an initial plasma creatinin concentration up to about 5 mg/100 ml the 150 l, 4 m² dialyzer could not bring creatinin back to high normal if the initial concentration were over 4 mg/100 ml.

The influence of membrane thickness is apparent from a comparison of Figs. 9 and 10 which latter gives the area volume curves for a halved membrane thickness. As was to be expected, the curves have shifted down and to the left.

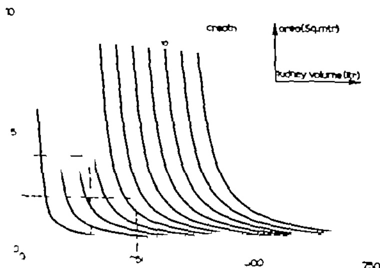


Fig. 10

Now the 250 l ½ m² kidney can normalize creatinin level if the initial level is not over 6.5 mg/100 ml the 150 l, 4 m² dialyzer can cope with initial levels of nearly 5 mg/100 ml or less

4. COMMENT

(a) Eq. (2) gives for any time t the total amount of the substance under consideration present in the two fluid compartments. This equation can be rearranged to

$$C_1 = C_{1_0} - \frac{(C_2 - C_{2_0})V_2 - \rho t}{V_1}$$

It follows that, provided V_1 and ρ are known, it is not necessary to take plasma samples to determine plasma concentrations during the dialysis session; they can be easily calculated from the outside concentrations which can, if so desired, be determined continuously [1].

For $C_{2_0} = 0$ the expression is further simplified to

$$C_1 = C_{1_0} - \frac{C_2 V_2 - \rho t}{V_1}$$ and for $C_{2_0} = 0$, $\rho = 0$ to

$$C_1 = C_{1_0} - C_2 \frac{V_2}{V_1}$$

(b) Another rearrangement of Eq. (2) yields

$$V_1 = \frac{V_2(C_2 - C_{2_0}) - \rho t}{C_{1_0} - C_1}$$

which for the $C_{2_0} = 0$, $\rho = 0$ case is further simplified to

$$V_1 = \frac{V_2 C_2}{C_{1_0} - C_1}$$

In physiological experimentation this expression can be used for the determination of the space of distribution of substances in the body. With the aid of the computer it is possible to calculate the parameters D, ρ, V_1 from Eq. (3) if at least 4 successive values of C_2 are known; the accuracy of these calculations increases with the number of successive C_2 values available.

---

[1]) In principle, plasma concentration could also be calculated from outside concentration with the aid of Eq. 1; in practice, however, this leads to insuperable difficulties.

SUMMARY

For a system consisting of two compartments separated by a membrane through which a solvent, present in both, can freely pass and where a solute, which is also able to pass through the membrane, is added at a constant rate to the fluid in one compartment, equations have been derived giving the concentrations, at any given time, of the solute in each compartment as function of compartment volume, solute concentrations at time zero, addition rate, specific permeation rate, and membrane area and thickness. The equations have been programmed on an IBM 7094 II computer. The computer results can be used to rationalize and optimalize haemodialysis procedures.

APPENDIX

D values for ureum and creatinin were determined in model experiments simulating the $C_{2_0} = 0$, $q = 0$ situation, where Eq. (1) reduces to the Fick equation

$$\Delta C = C_{1_0} \cdot e^{-Bt}$$

from which

$$\log \Delta C = \log C_{1_0} - Bt/2.3$$

$$B = \frac{2.3\,(\log C_{1_0} - \log \Delta C)}{t} \quad \text{so}$$

$$D' = \frac{2.3\,(\log C_{1_0} - \log \Delta C)\; V_1 V_2}{t(V_1 + V_2)}$$

$$D = \frac{2.3\,(\log C_{1_0} - \log \Delta C)\; V_1 V_2 \Delta x}{S\, t(V_1 + V_2)}$$

Both compartments of the model were closed circuits through which fluid circulated. Part of the V_1 circuit consisted of a length of cellulose tubing (Visking) wrapped around the inner of two *concentric cylinders*; the space between the cylinders served as V_2. V_1 was filled with plasma, V_2 with Sterofundin (Braun-Melsungen). The model was placed in a water bath at 37° C ± 0.1. *Creatinin* determinations were made according to Jaffé[4]; for ureum the auto-analyser method was used. To determine V_1 and V_2 known amounts of creatinin were introduced into the compartments, and their concentrations were determined after complete mixing had occurred, i.e. after one minute at which time diffusion effects are negligible. V_1 and V_2 were calculated from the concentration increase, and the concentration, respectively.

The *D* values found were 0.25×10^{-4} for creatinin ($s \sim 0.015$ for $n = 5$) and 0.38×10^{-4} for ureum ($s \sim 0.022$ for $n = 6$).

Acta Physiol Pharmacol Neerl 14 (1966) 220–230 © *North-Holland Publ. Co.*

NETHERLANDS SOCIETY FOR PHYSIOLOGY AND PHARMACOLOGY

PROCEEDINGS OF THE MEETING WITH THE BELGIAN SOCIETY FOR PHYSIOLOGY AND PHARMACOLOGY AMSTERDAM NOVEMBER 5 AND 6 1965

PROGRAMME

No abstracts received of the following papers:

KAMMEN F VAN and D DE WIED Dissociation between antidiuretic and vasopressor activity in rat posterior pituitaries upon incubation *in vitro*

ERNST A M and P G SMELIK The extra pyramidal point of attack of gnawing inducing compound

SMELIK, P G and A M ERNST The role of dopaminergic pathways in the gnawing syndrome of the rat

G W A E le Coultre Mulder C A Veldhuizen and J Bouman *Growth experiments on human cells (T Amnionic and HeLa cells) in fluoride containing media*

Department of Physiology University of Leiden

Fluoride is reported to depress growth of human HeLa cells and mouse fibroblasts at concentrations as low as 0 045 ppm (BERRY and TRILLWOOD 1963). Human plasma fluoride is constant at a level of 0.2 ppm (SINGER and ARMSTRONG 1960) but will be augmented near fluoride deposits. As cells might differ in their sensitivity to fluoride ions, we used *in vitro* cultures of cells derived from human kidney (T cells) from human uterine cervical carcinoma (HeLa cells) and fast growing cells derived from human amnion. Growth curves were determined by estimating the total amount of DNA in every culture flask. All cells were grown in duplicate at 37° during 2, 3 4 5 6 and 7 days in 5×5×16 cm closed culture flasks containing 0 1 2 4, 8 and 16 ppm of fluoride Each flask contained 13 ml of culture medium with initially approximately 600 000 cells. Hanks medium supplemented with 5 % calf serum and with lactalbumin, penicillin and streptomycin was used. The medium was changed every 48 hours. When DNA determinations were to be made the medium was removed and the cell layer was washed once with saline The cells were loosened from the glass surface with a solution of 0.25 % trypsin and centrifuged. DNA determinations in duplicate (T and Amnionic cells) were made according to the method of CERIOTTI (1952) against standard solutions of purified thymus DNA. Depression of DNA synthesis at different fluoride levels relative to control cultures was 2 % at 1 ppm F (n.s.) 3 % at 2 ppm F (n.s.) 6 % at 4 ppm F (n.s.) 18 % at 8 ppm F ($P \sim 0.001$) and 23 % at 16 ppm F^- ($P < 0.001$). This inhibition did not differ significantly among the three types of cells. These results confirm the work of others (PROFFITT and ACKERMAN 1964 ALBRIGHT 1964 and ARMSTRONG *et al.*, 1965) with various cell cultures, but differ from those of BERRY and TRILLWOOD (1963).

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ARMSTRONG, W D *et al.*, Brit. med. J 1 486 (1965).

BERRY R. J and W TRILLWOOD Brit. med J 2 1064 (1963).
CERIOTTI G J biol Chem. 198 97 (105)
PROFFIT W R. and J L ACKERMAN Science 145; 932 (1964)
SINGER L. and W D ARMSTRONG J appl Physiol 15; 508 (1960)

E D Gerlings L N Bouman and G Jambroes *The action of the autonomic nerves on atrial contractility*

Department of Physiology University of Amsterdam

We have shown previously that stimulation of the vagal nerves has no direct influence on the isovolumetric contractility of the ventricles of the isolated rabbit heart if the frequency of the heart is kept constant by electrical pacing of the right ventricle (GERLINGS *et al* 1965) Stimulation of the right accelerator nerve however causes a marked augmentation of the contractility of both ventricles.

In order to investigate whether the contractility of the atria is changed directly by the action of the autonomic nerves in the isolated retrogradely perfused rabbit heart the atrial contractility was measured isovolumetrically by introducing a rubber balloon into the cavity of the left atrium via a small incision in the apex of the left ventricle The balloon was filled with water until a diastolic pressure of 5 cm H_2O was reached and then connected to an Elema pressure transducer

A second balloon was introduced into the cavity of the right ventricle In the spontaneously beating heart stimulation of both vagal nerves caused a marked decrease in both atrial and ventricular contractility If however the cardiac frequency was kept constant a decrease in contractility was observed only in the atrium

On the other hand stimulation of the right accelerator nerves caused a marked increase in the maximum systolic pressure in both atrium and ventricle Even when the heart was driven at such a high frequency that no further increase in frequency occurred during accelerator stimulation a positive inotropic effect of the accelerator nerves on both atrial and ventricular myocardium was still present.

Frequently we observed that the increase in atrial contractility was preceded by a transient decrease This negative effect was completely abolished by adding atropine sulphate (3 μg/ml) to the perfusion fluid This observation supports the assumption of

Huković (1959) that cholinergic fibres are present in the accelerator nerves of the rabbit which are responsible for this inhibitory effect.

From our experimental results we have reached the conclusion that a direct antagonistic action of the para and orthosympathetic nerves on the heart is only present in the atrial portion of the heart, whereas solely the orthosympathetic nerves influence directly ventricular contractility

REFERENCES

Geerlings, E. D. L. à Bouman G. Jamsbors and P. A. Biersteker, Acta Physiol. Pharmacol. Neerl. (in the press).
Huković, S., Brit. J. Pharmacol. Chemotherap. 14; 372 (1959).

N. Hyppolito Jurkiewicz and A. Jurkiewicz *Cholinergic drugs tested on the rat fundus strip*

Dept. of Pharmacology University of Nijmegen

Cumulative dose-response curves obtained with cholinergic drugs on the rat fundus strip seem to be the resultant of two different dose-response curves and therefore two different components in the action of these drugs. Analysis by means of anticholinergic drugs showed that both components in the action can be blocked. The two components in the dose-response curve however behaved differently. The antagonism of the first component of the curve manifested itself as a decline in the slope, while in the second component of the curve a parallel shift was found. The assumption was made that the first component might be the result of some indirect (e.g. liberation of serotonine or acetylcholine) the second component of a direct (muscarinic) action. This assumption was put to a number of tests which were discussed.

C. van der Meer *Tourniquet shock*

Pharmacological Laboratory University of Amsterdam

Tourniquet shock in rats is characterized by a loss of about 0 ml fluid into the hindlegs and an initial fall in blood pressure to 60–75 mm Hg. Shortly before death a second fall in blood pressure is found accompanied by arrhythmia and ECG changes similar

to those found in K intoxication Plasma K rises steadily to reach values of 10–13 meq/l just before death While 100 % of non-deficient rats die within 4–6 h K-deficient rats show a 39 % 24 h survival

Fluid loss and initial fall in blood pressure are not primary causes of death since survival rate is not correlated with magnitude of fluid loss fluid loss and initial fall in blood pressure are identical in K-deficient rats (30 % survival) and in non-deficient rats (0 % survival) dehydration and reduction of blood pressure in normal rats to levels identical to those found in tourniquet shock does not cause a comparable mortality

The following sequence of events leads to death The tourniquets cause anoxia of the hindlegs, followed by capillary damage vasodilatation and necrosis of the tissues Capillary damage causes fluid loss and haemoconcentration Fluid loss and vasodilatation cause the initial fall in blood pressure The necrotic muscles release K and take up Na Haemoconcentration and reduced blood pressure inhibit renal excretion of the excess K plasma K rises while plasma-Na falls This ionic imbalance causes functional heart damage leading to the terminal fall in blood pressure and to respiratory standstill at about 30 mm Hg

W J Rietveld and W E M Tordoir *Phase control of the locomotor activity of the rabbit*

Psychophysiology Division Department of Physiology University of Leyden

In the course of an investigation into the influence of illumination on circadian activity rhythms the effect of illumination upon the locomotor activity of the rabbit was studied

Three variables were investigated *vi* light-dark ratio light intensity level and twilight duration

It was found that under conditions of natural illumination the animals exhibit one activity peak at dawn and another one at dusk, superimposed upon a basic activity Under constant artificial illumination this basic activity is highest during the night and lowest during the daytime hours Experiments in which the light dark ratio was varied showed that the moment of light-off is the principal Zeitgeber

It was also found that the dusk activity peaks depend on the dawn peaks. There was no systematic influence of illumination level either on the phase of the basic activity or on peak height.

In young animals only dawn and dusk activity peaks decrease with increase of twilight duration.

It can be concluded that the most important factor in phase control is the moment of intensity decrease (dusk)

W Storm van Leeuwen A Kamp M L Kok and A M Tielen *Relations between behaviour in animals and electrical brain activities*

Institute of Medical Physics T.N.O., Utrecht

By means of 8-channel radiotelemetering of the EEG in dogs with chronically indwelling electrodes it has been possible to study electrical activities in various parts of the brain in the freely moving animal. The electrical activities and a verbal report of the animal's behaviour were recorded on an electro-encephalograph and on a multichannel taperecorder On the ground of certain characteristics of the electrical phenomena, i.e. frequency waveform, location and amplitude and by virtue of relations with behavioral aspects, a number of activities were distinguished which were called specific activities" They are the following

1 Alpha rhythms in visual cortex, with frequencies of 10–14c/sec and amplitudes up to 600 μV occurring at eye closure.
2 Lambda waves in visual cortex with wave durations of 1/5–1/7 sec amplitudes up to 600 μV mono- and biphasic associated with eye movements made while the dog is "looking with interest" in bright light
3 Beta rhythms in visual cortex with frequencies of 25–35 c/sec, amplitudes up to 300 μV occurring while the dog has its eyes open in bright light but is not "looking with interest"
4 Beta rhythms in ectosylvian cortex with frequencies of [illegible]0–25 c/sec, amplitudes up to [illegible]00 μV occurring while the animal is "looking vacantly"
5 Theta rhythms in hippocampus with frequencies of 5–7 c/sec, amplitudes up to 400 μV occurring while the dog is "walking to a goal" or is "tense"

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E. *Beta rhythms in olfactory bulb coincide* with beta rhythms in ectosylvian cortex. They *alternate* with lambda waves and hippocampal theta rhythms.

It is suggested that these relations provide an indication of the animal's direction of attention.

H J Th Thalen and Jw van den Berg *Threshold measurements and electrodes of the cardiac pacemaker*

Laboratory of Medical Physics University of Groningen

We developed our first pacemaker in 1960 On account of experiences with this first specimen and new investigations, a new pacemaker with new leads and electrodes has been developed since the spring of 1964

INVESTIGATIONS

In mongrel dogs (15–25 kg) a heart block was made by ligating the bundle of His (method after Dr Homan van de Heide)

Electrodes were placed on or in the ventricles and elsewhere in the body (indifferent electrode) The leads perforated the dog's skin near the neck and were connected to a pacemaker For the measurements our Cardio Test was connected. Many electrode combinations could be tested this way

RESULTS (Table 1)

1 Monopolar stimulation (one electrode in the heart, the other somewhere else in the body) gives optimal results when the negative pole of the pacemaker is connected to the heart-electrode and the positive pole to the indifferent electrode

2 The difference between optimal monopolar stimulation and bipolar stimulation (= both electrodes in the heart) is not significant

3 Every heart-electrode has the same charge-threshold regardless of the choice of the indifferent electrodes. This means that a certain heart-electrode always needs the same amount of charge to induce a contraction of the heartmuscle.

6 Irregular activities in amygdala with frequencies between *10–20 c/sec* amplitudes up to 300 μV occurring while the animal is sniffing

7 Beta rhythms *in olfactory* bulb at 35–40 c/sec amplitudes up to 600 μV occurring when the animal is expiring through its nose

8 Theta and delta rhythms in olfactory bulb with frequencies of 5–6 and 1–3 c/sec amplitudes up to 500 μV associated with the animal's type of breathing

W Storm van Leeuwen A Kamp F H Lopes da Silva and F de Quartel *Mutual relations of specific activities*

Institute of Medical Physics T N O Utrecht

The specific activities occur during certain types of behaviour This entails that specific activities do not take place during some other types of behaviour which themselves may have a relation with another specific activity From this it follows that the specific activities can also be studied in their mutual relations, irrespective of possible relations with behaviour It was found that some specific activities often occur and simultaneously coincide whereas others rarely take place at the same time but alternate From the mutual relations which have been observed the following are mentioned

A. *Alpha rhythms in visual cortex* may *coincide* with alpha rhythms occurring in other structures (lat geniculate body medial *thalamic structures) Alpha rhythms alternate* with lambda waves, visual cortex beta rhythms and hippocampal theta rhythms

B *Lambda waves in visual cortex* may *coincide* with hippocampal theta rhythms Lambda waves *alternate* with visual cortex beta rhythms and with amygdalar 10–20 c/sec irregular activities

C *Beta rhythms in visual cortex alternate* with lambda waves and with hippocampal theta rhythms

D *Irregular 10–20 c/sec activities in amygdala coincide* with 5–7 c/sec activities in olfactory bulb and *alternate* with lambda waves and hippocampal theta rhythms.

CONCLUSIONS

1 The threshold is determined by the type of the negative electrode. The indifferent electrode is only needed to complete the electrical circuit.

2 The threshold at a given pulse-duration is determined by a critical value of the current-density at the intact heartmuscle around the negative electrode. When this density is achieved, the depolarization of the heartmuscle arrives at a critical value resulting in a contraction of the heart.

The increase of the threshold can be explained accordingly Around the electrode connective tissue is formed. The resistance remains almost equal, but the result is an increase of the distance between the electrode and the intact heartmuscle. The current density decreases *approximately* quadratic with the *increase* of the distance To achieve the same current-density at the intact heart muscle, more current and thus more charge is needed, requiring a higher voltage of the pulse.

STIMULATING CIRCUIT

Considering these results, we developed for the *Groningen pace maker* a monopolar stimulating circuit with a large stainless steel plate in the wall of the pacemaker as *indifferent electrode*, making the indifferent electrode unbreakable Because of the great surface the current-density is very low so that stimulation of nearby nerves or muscles does not occur

The heart-electrode is connected by a lead to the pacemaker The *lead* (Fig 1A) should be unbreakable, flexible extensible well isolated accepted by the human body of the same metal as the electrode to avoid contact potentials and corrosion, and should have a low resistance. For our lead we took a thin platinum 90-iridium 10 % spiralled wire Mechanical stability is achieved by embedding the spiral completely in silicon rubber The great advantage of this construction is the core of silicon rubber inside the spiral Because of this core the lead cannot be flattened. After great external deformation the lead immediately recovers completely In this way predilection-places for breakage are avoided.

Four our *heart-electrode* we wanted to lengthen the spiral into the myocardium and to maintain the mechanical stability Therefore a U formed electrode (Fig 1B) was constructed with a plati-

TABLE 1

Monopolar and bipolar stimulation

		electrode of the heart	− +	*threshold voltage* in Volt	*threshold charge* in micro-Coulomb	*operational charge* in micro-Coulomb
I	1	Elmqvist/Senning (F)	E–I	0.8	2.5	8
			E–Ch	1	2	38.5
	2	Chardack (Ch)	Ch–E	0.55	1	1
			Ch–I	0.5	1	22
	3	Pin (P)	P–I	0.75		22
			P–Lo	0.7	2	24
	4	*Lagergren (Lo)* catheter	Lo–P	0.85	2.5	4.5
			Lo–I	0.75	2	27
	5	St George (St G)	St.G–I	0.75	2.25	24.5
			St.G–U	1.1	2.5	19.5
	6	Groningen electrode	G–St G	0.65	1.5	18.5
			G–I	0.7	1.5	19
	7	Groningen electrode (G_1)	G_1–I	0.9	2	20.5
			G_1–G_2	1		21.5
	8	Groningen electrode (G_2)	G_2–G_1	1.8	4	22
			G_2–I	1.4	4	21.5
	9	Groningen electrode (G_2)	G_2–I	1.7	7	8.5
			G_1–G_2	6	7	1.5
	10	Groningen electrode (G_2)	G_2–G_1	1.6	3	22.5
			G_2–I	1.3	2.5	8

20 combinations in 5 dogs of 10 heart-electrodes and 5 indifferent electrodes (I) subcutaneous 4 i.c.s.
Heart-electrodes – I = unipolar stimulation
Heart-electrodes – heart-electrodes = bipolar stimulation
Pulse-duration 2 msec; pulse shape rectangle; frequency 95/min

4. The threshold value depends on the pulse-duration but for a certain pulse-duration it is independent of the location or the type of the indifferent electrode

5 In long term experiments increasing thresholds are sometimes seen but these thresholds remain independent of the indifferent electrode



Fig. 1

num 90-iridium 10 % core in the spiral The electrode is now completely composed of one metal The electrode enters into an incision previously made in the myocardium After the implantation the heartmuscle regenerates through the loop of the electrode thus fixing it in a natural way in the myocardium (Fig 10)

O L Wolthuis and E Meeter *An analysis of problems met in the therapy against anticholinesterase poisoning*

Medical Biological Laboratory RVO-TNO Lange Kleiweg 139 Rijswijk Z.H

Male rats weighing 180–200 g of an inbred laboratory strain were injected with DFP (4 × 8 × or 16 × LD50 [1]) s.c) and atropine (10 mg i p) Artificial respiration was started immediately after injection Before injection of DFP the ECG heart frequency blood pressure respiratory minute volume and neuromuscular function during tetanic stimulation (25 50 100 and 200 stim./sec.) of the gastrocnemicus-soleus muscles and of the diaphragm were recorded (control tests)

Sixty minutes after DFP injection a second testrun took place and immediately thereafter either P2S or LüH₆ (Toxogonin®), both in a dose of 100 mg/kg i p was injected. Fifteen minutes after oxime injection the tests were repeated to establish recovery of function The experiments were continued by recording the parameters mentioned above every 30 minutes.

The results from these experiments indicate that

1) both oximes are highly effective in counteracting neuromuscular inhibition
2) both oximes are less effective in restoring central inhibition caused by DFP the differences found between the effectiveness of both oximes do not plead in favour of the use of LüH₆
3) irrespective of oxime-therapy and even if oximes were left out completely heart failure occurred several hours after DFP injection the latency depending on the dose of DFP-used. Recent experiments on isolated rat hearts indicate that very likely the above-mentioned effects of DFP on the heart cannot be influenced by atropine either

[1]) LD50 was determined in atropinized anaesthetized rats of the same strain and body weight

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Department of Physiology, University of Leiden

CONTRIBUTION OF ALVEOLAR SURFACE LINING TO LUNG MECHANICS

ANALYSIS OF IN VIVO MEASUREMENTS OF AIR- AND FLUID-FILLED LUNGS

BY

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we made a rough estimate of the thickness of the surface lining With this thickness we calculated the time in which thermodynamic equilibrium occurred in the surface lining

If such an equilibrium should exist surface tension of the surface lining could not contribute to the hysteresis of the P V diagrams of air-filled lungs.

To check this theory excised lungs of the same dogs were used for in vitro P V measurements with air before and after the removal of surface active material by lavage.

So the purpose of this investigation was two-fold

1 to calculate the contribution of the surface tension of the surface lining to lungwork in vivo
2. to verify whether the surface lining can contribute to the hysteresis of the P V diagrams.

2. MATERIAL AND METHODS

14 Mongrel dogs of ages between one and four years and weighing from 14–18 kg were used of which the first four were used to test procedures and techniques of pressure and surface tension measurements.

In the dogs to be subjected to lung lavage a permanent tracheostoma was made several days before the experiment proper in order to obviate the need of intubation.

A flexible and radiopaque endobronchial tube was introduced through this tracheostoma into the left main bronchus and kept in position by means of an inflatable cuff

The tube could be connected either to a 50 ml syringe allowing the left lower lobe to be filled with air in 50 ml steps or to a three-way system by means of which the tube could be connected either to a calibrated container from which the lavage fluid could flow into the lobe or to a calibrated cylinder into which the fluid could be siphoned (see Fig 1)

As lavage fluid of the same osmolarity as dog plasma Sterofundin (Braun Melsungen) at body temperature was used. If necessary sedatives were given to the dogs.

Pressures in the tube were continuously recorded with a Statham pressure transducer on a Grass polygraph. For all experiments the pressure transducer was kept at the same level i.e. the dog's spine.

1 INTRODUCTION

In 1929 von Neergard showed that a large part of the retractive force of *excised dog lungs* can be removed by degassing them and filling them with a gum arabic saline solution.

Since then several investigators performed similar experiments, most of which were done on excised lungs (RADFORD 1954 1957 MEAD *et al* 1957 CLEMENTS *et al.* 1958)

In doing so they suggested to eliminate all but a small fraction of the surface free energy of these lungs. They concluded that *surface tension played a very important role in lung mechanics.* A more recent discovery in lung mechanics concerned the surface lining of lung alveoli with a film of lipoprotein (PATTLE 1955 1965) The lipid fraction of this film might have the function of lowering the surface tension and might contribute to the hysteresis of lung compliance (BROWN *et al.* 1959 BONDURANT 1960 MEAD 1961) Most of the methods of studying surface lining were done *on extracts of excised lungs.*

In our laboratory KYLSTRA (1960) and DE BOER (1963) demonstrated an easy way of filling one lung of an unanaesthetized dog with an isotonic Ringer solution

Thus it was also possible to make static *P V* diagrams of fluid filled lungs.

From the static *P V* diagrams of air filled lungs we have calculated *the P V diagrams of these lungs if filled with fluid* These calculated *P V* diagrams were compared with the experimentally found *P V* diagrams of the fluid filled lungs.

Because surface tension was neglected in the calculations this comparison served as to estimate the pressure part of the surface tension to lungwork

These *P V* diagrams however are a summation of lung and thoracic wall compliances. Therefore additional experiments with the same but anaesthetized dogs with open thorax were made to separate these phenomena.

With the technique mentioned it was also possible to measure the surface tension of the fluid which could be siphoned from the lungs *A quantitative analysis* of the lipid and protein fraction of the surfactant was made afterwards

From these data and the geometrical structure of the dog lung

genized so there could not be any air left even in the smallest air-spaces. This volume was also measured in the same cylinder The difference between those volumes had to be the residual air volume at end-inspiratory apnea at zero pressure. This volume approximated the amount of fluid residue remaining in the air spaces after the lavage, viz. 60–100 ml, dependent on the dog's weight.

In order to get comparable *P V* diagrams of air and fluid-filled lobes the air and fluid residual values were assumed to be equal. Starting from the residual volume the lobe was first filled with air in 50 ml steps. This was done twice in order to get two *P V* diagrams and was followed by the lavage procedure.

Lung lavage was discussed in detail elsewhere (DE BOER, 1963) When lavage fluid did not contain air bubbles anymore the lobe was filled in 50 ml steps up to 250 ml from the residual volume and then emptied again also in 50 ml steps, while pressures were recorded simultaneously The air as well as the fluid filling procedure each took about 50 min.

In the way just described P V diagrams could be made of air and fluid filled lobes respectively

To separate lung and thoracic wall compliance and to investigate whether or not surface lining might contribute to the hysteresis of lung compliance the following experiments were added.

In order to be certain the lavage fluid residue had been completely absorbed, the air filling procedure of the same but now anaesthetized dogs with open thorax (2 ribs removed) was repeated one week later The heterolateral lungs were ventilated by intermittent positive pressure breathing. Then these dogs were sacrificed and their left lower lobes were excised. Of these excised lobes *P V* measurements with air were made after which these lobes were lavaged with Sterofundin until all surface active material had been removed. This could be checked by *measuring surface* tension of the siphoned lavage fluid. In conclusion *the air-filling* procedure was repeated.

Between the successive compliance measurements of the unanaesthetized dogs the left lower lobe was lavaged with different amounts of Sterofundin.

In order to get some idea about the amounts of surfactant which could be found in the more peripheral parts of the lung the left

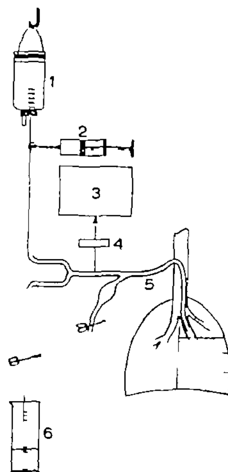


Fig 1

Diagram of lung lavage 1 electrically heated container for lavage fluid, 50 ml syringe 3 Grass polygraph, 4 Statham pressure transducer 5. flexible and radiopaque endo-bronchial tube 6. calibrated reservoir

If the lavage fluid had the same osmolarity as dog plasma a certain amount of fluid residue remained in the left lower lobe after the lavage

In order to determine the amount of air remaining in this lobe at zero pressure (1 ata) the trachea was clamped at end inspiratory apnea of 3 anaesthetized dogs, after which a thoracotomy was performed so as to remove the left lower lobe, the bronchus of this lobe being clamped and the lobes volume was measured in a calibrated cylinder filled with Sterofundin

Then the air from the lobe was released and the lobe was homo-

curve was nearly identical with the former one (see Fig. 2). All the $P\ V$ diagrams of the air filled excised lobes were practically identical to each other before and after removal of surfactant by lavage (see Fig. 3) Each loop has a cycle of about 50 min.

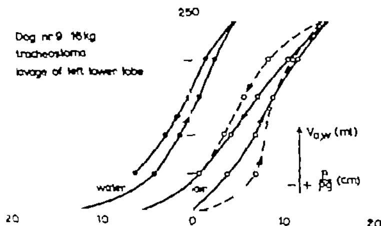


Fig. 2.

Air (o—o) and fluid (●—●) $P\ V$ diagrams of the left lower lobe of a dog with closed thorax and an air $P\ V$ diagram ( ) of the same lobe of the same dog with open thorax.

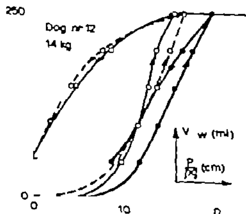


Fig. 3.

$P\ V$ diagrams of an excised left lower lobe before (o—o), during (●—●), and after (●) the removal of surfactant by lavage.

lower lobe was filled with a 250 ml portion and after 15 min emptied again in 50 ml steps assuming the first siphoned 50 ml to come from the central parts of the lung and the last 50 ml from the peripheral parts.

The rate of production of the surfactant was roughly estimated by keeping 100 ml portions in the lung for periods of 2½ 5 and 10 min

Of these siphoned portions the surface tension was measured and a quantitative analysis of the lipid and protein fraction was made In another series of experiments the freeze-dried material of 1000 ml portions was dissolved again in different amounts of Sterofundin varying from 2½–640 ml

Then the surface tension of these diluted portions was measured and these surface tension values were plotted against the lipoprotein concentration of the corresponding portions.

In order to find out the total amount of surfactant in a dog's left lower lobe the lipoprotein content of the siphoned lavage fluid of the first 40 min of lung lavage was estimated and reduced by the production of surfactant during this time

Surface tension measurements were made by a du Nouy balance at 37 °C ± 1 °C

The accuracy of this balance was checked by measuring well known surface tensions of several substances.

The lipid content was estimated by freeze-drying and extracting the dry material with chloroform methanol (FOLCH 1957)

The protein content was estimated by the biuret method (GORNALL *et al.* 1949)

3 RESULTS OF EXPERIMENTS

3 1 COMPLIANCE MEASUREMENTS OF AIR AND FLUID FILLED LEFT LOWER LOBES

All the P V diagrams of air filled lobes of unanaesthetized dogs showed the same qualitative alterations when the lobe was filled with fluid shifting of the hysteresis loop to the left narrowing and changing of direction of this loop (see Fig 2)

All the P V diagrams of air filled lobes of anaesthetized dogs with open thorax were different as to the initial part of the ascending curve up to 150–200 ml and after this point the ascending

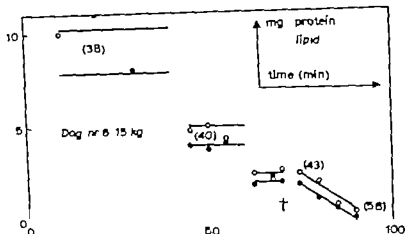


Fig. 5.

Approximation of the rat of production of surfactant by keeping 100 ml portions of lavage fluid in a dog left lower lobe for 10 5 and 2½ min resp. during lif and for 5 min after death. Average surface tension f these portions between brackets.

portions were plotted against the lipoprotein concentrations of the corresponding diluted portions (see Fig 6)

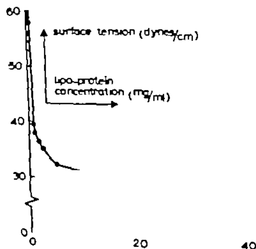


Fig. 6.

Surface tensions measurement plotted against lipoprotein-concentrations of the corresponding diluted portions.

3 2 SURFACE TENSION MEASUREMENTS AND QUANTITATIVE LIPOPROTEIN DETERMINATIONS OF THE SIPHONED LAVAGE FLUID

The average *ST* of Sterofundin amounted to 58 dynes/cm at 37 C *ST* of the siphoned Sterofundin was markedly lower than *ST* of fresh Sterofundin while it contained considerable amounts of lipoprotein

Of the 50 ml steps of the 250 ml portions the average *ST* decreased and the lipoprotein concentration increased (see Fig 4)

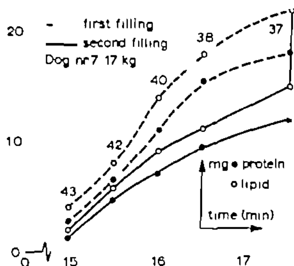


Fig 4

Filling of the left lower lobe with 250 ml portions for 15 min and emptying it by 50 ml steps. Surface tension of the first filling

This decrease of *ST* and increase of lipoprotein concentration was found at any time even after 4 hours of lung lavage. When the dog was sacrificed however the *ST* of the samples increased to the average *ST* value of fresh Sterofundin whereas the lipoprotein concentration decreased to about zero within 20 min after the death (see Fig 5)

The *ST* and lipoprotein concentration of the 100 ml portions which were kept in the lung for different periods are shown in Fig 5 As can be seen the production rate of surfactant in the left lower lobe will be about 1¼ mg/min.

The *ST* of the dissolved freeze-dried material of the 1000 ml

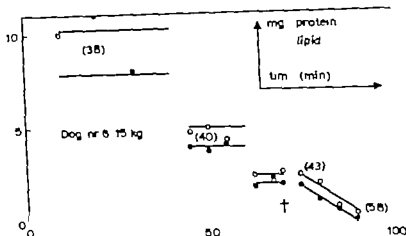


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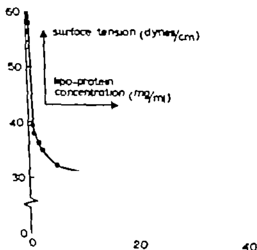


Fig. 6.

Surface tensions measurement plotted against lipoprotein-concentrations of the corresponding diluted portions.

The average total amount of lipoprotein of the siphoned lavage fluid of the first 40 min of lung lavage reduced by the production of surfactant during this time amounted to 160 mg

The standard deviation of the *ST* measurements amounted to 2 dynes/cm for $n = 50$

The standard deviation of lipid and protein determinations amounted to 0 5 mg/100 ml and 0 4 mg/100 ml resp for $n = 10$ *The within dog variance of the ST measurements and lipo*-protein determinations was very small the 'between dog vari ance was somewhat higher

4. RESULTS OF CALCULATIONS

4 1 CONTRIBUTION OF SURFACE TENSION TO LUNG WORK

Before calculating *P V* diagrams of fluid filled left lower lobes from air filled lobes of living dogs with closed thorax some sim plifying assumptions had to be made

1 At all levels the lobe has the same cross section
2 During filling the left lower lobe with Sterofundin the horizontal section increases while the height of the lobe remains the same.
3 The fluid filled lobe is empty as soon as the hydrostatic pressure in the lowest part of the lobe falls below the atmospheric pressure

First of all we had to calculate the ascending part of the loop caused by filling the lobe with fluid

Let h_L be the height of the lobe h_0 be the height up to where *the lobe is filled with fluid* h *be the rising co-ordinate (see Fig 7)*

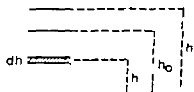


Fig 7

Model of a dog's left lower lobe

At h the hydrostatic pressure $p = \varrho g(h_0 - h)$ where ϱ represents *the density of the fluid and* g *the specific gravity*

If the whole lobe were at this pressure the volume increase

would be $V_{air}(p)$. However this is only the case with the small part dh and hence the volume increase of this part becomes

$$dV_{water} = \frac{dh}{h_L} \quad V_{air}$$

The volume increase of the whole fluid-filled lobe will be found by integrating this equation

$$V_{water} = \frac{1}{h_L} \int_0^{h_L} V_{air} \quad dh$$

and by substituting $dp = -\varrho g dh$ and by changing the integration boundaries into $p = \varrho g h_0$ at $h = 0$ and $p = \varrho g(h_0 - h_L)$ at $h = h_L$ the integral becomes

$$V_w = -\frac{1}{\varrho g h_L} \int_{\varrho g h_0}^{\varrho g(h_0 - h_L)} V \quad dp = \frac{1}{\varrho g h_L} \int_{\varrho g(h_0 - h_L)}^{\varrho g h_0} V \quad dp \qquad (1)$$

According to assumption 2, $V = 0$ if $h_0 - h_L < 0$

$$V_w = \frac{1}{\varrho g h_L} \int_0^{\varrho g h_0} V \quad dp. \qquad (2)$$

Grafically this integral represents the area beneath $V - (p/\varrho g)$ curve between $p = \varrho g(h_0 - h_L)$ and $p = \varrho g h_0$ for eq. (1) and between $p = 0$ and $p = \varrho g h_0$ for eq. (2) (see Fig. 8)

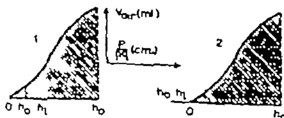


Fig. 8.

Graphical illustration of eqs (1) and (2).

This area divided by h_L gives V. In our dogs h_L amounted to 10–15 cm. Intrapulmonary pressure in fluid filled lobes depends

The average total amount of lipoprotein of the siphoned lavage fluid of the first 40 min of lung lavage reduced by the production of surfactant during this time amounted to 160 mg

The standard deviation of the *ST* measurements amounted to 2 dynes/cm for $n = 50$

The standard deviation of lipid and protein determinations amounted to 0 5 mg/100 ml and 0 4 mg/100 ml resp for $n = 10$ The within dog variance of the *ST* measurements and lipoprotein determinations was very small the 'between dog variance was somewhat higher

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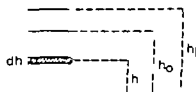


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If the whole lobe were at this pressure the volume increase

would be $V_{air}(p)$ However this is only the case with the small part dh and hence the volume increase of this part becomes

$$dV_{water} = \frac{dh}{h_L} \; V_{air}.$$

The volume increase of the whole fluid filled lobe will be found by integrating this equation

$$V_{water} = \frac{1}{h_L} \int^{h_L} V_{air} \; dh$$

and by substituting $dp = -\varrho g dh$ and by changing the integration boundaries into $p = \varrho g h_0$ at $h = 0$ and $p = \varrho g(h_0 - h_L)$ at $h = h_L$ the integral becomes

$$V_w = -\frac{1}{\varrho g h_L} \int_{\varrho g h_0}^{\varrho g(h_0 - h_L)} V_{air} \; dp = \frac{1}{\varrho g h_L} \int_{\varrho g(h_0 - h_L)}^{\varrho g h_0} V_{air} \; dp \qquad (1)$$

According to assumption 3 $V_{air} = 0$ if $h_0 - h_L < 0$

$$V_w = \frac{1}{\varrho g h_L} \int^{\varrho g h_0} V_{air} \; dp \qquad (2)$$

Grafically this integral represents the area beneath $V_{air} - (p/\varrho g)$ curve between $p = \varrho g(h_0 - h_L)$ and $p = \varrho g h_0$ for eq (1) and between $p = 0$ and $p = \varrho g h_0$ for eq. (2) (see Fig 8).

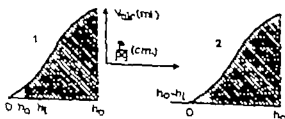


Fig. 8.

Graphical illustration of eqs. (1) and (2).

This area divided by h_L gives V_w In our dogs h_L amounted to 10–15 cm. Intrapulmonary pressure in fluid filled lobes depends

on the height of the fluid column above or beneath the pressure transducer which leads to the so-called shift to the right or to the left respectively Therefore we can shift the experimentally found curve of the fluid filled lobe to the origin of the plots.

From the P V diagrams of air filled lobes it was then possible to calculate the ascending curve of the hysteresis loop of the lobes if fluid filled From Fig 9 it appears that the curve does

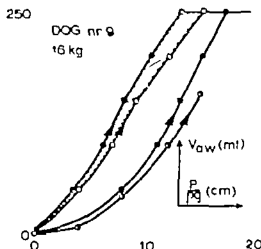


Fig 9

Calculation of the ascending curve of a P V diagram of a fluid filled lobe from the ascending curve of an experimentally found P V diagram of an air filled lobe and calculation of the pressure part of the surface tension (shaded area) of the surface lining (o——o) experimental air curve (•——•) experimental fluid curve (⊕——⊕) calculated air curve (◑——◑) calculated fluid curve

not continue up to 250 ml because at about 160 ml fluid the lowest parts of the lobe are already stretched to the same extent as when the lobe is filled with 250 ml air so with the given data it is impossible to calculate the curve at a pressure higher than 15 cm water

As the fluid filled lobe is unequally stretched at any level it is far more complicated to calculate the descending curve of the hysteresis loop of a fluid filled lobe from an air filled lobe At siphoning the lobe any level follows its own hysteresis curve

For $h = 0$ pressure is maximal so $p = \rho g h_0$. The volume decrease for the small part dh will be again $dV_w = (dh/h_L)\ V$

According to the hysteresis curve V_a will now decrease Fig 10

a 3-dimensional graphic, illustrates the calculation of such a curve where the triangles represent V_w. Figs. 9, 10 and 11 are based on Fig. 2

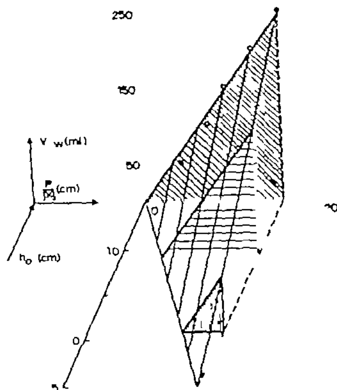


Fig. 10.

Calculation of the descending curve of a P V diagram of a fluid filled lobe from the ascending curve of an experimentally found P V diagram of an air-filled lobe. For simplicity lines are drawn straight.

Fig. 11 shows a hysteresis loop of a fluid filled lobe calculated from a hysteresis loop of an air filled lobe compared with the one found experimentally.

The calculated and experimental loops much resemble one another both qualitatively and quantitatively. However a small discrepancy remains: the higher the pressures the greater this

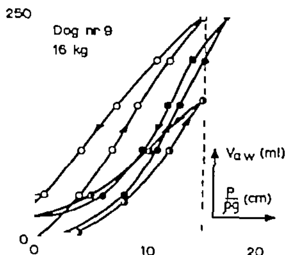


Fig 11

Comparison of the experimentally (•—•) found and calculated (o—o) hysteresis loops of a fluid filled left lower lobe

discrepancy while the calculated pressures are always higher than those found experimentally This is probably due to the rough assumptions and the neglect of the small surface tension

Differentiation on the ascending curve of the fluid P V diagram which was found experimentally gives a P V diagram of an air filled lobe without surface tension The area between these calculated and experimental ascending curves of the air P V diagrams appears to be the contribution of the surface tension to lung work (see Fig 9)

As can be seen from this figure the probable contribution of surface tension to lung work amounted to about 12 % The range of these contribution values varied from 5–15 % with an average of 11 % for $n = 7$ However we consider the accuracy of these values of the same order of magnitude

4 2 Contribution of surface lining to compliance hysteresis

It has been suggested that variations in surface tension of the surface lining could contribute to the hysteresis phenomenon of P V diagrams of air filled lungs

The surface tension depends on the number of surface active molecules which are adsorbed at the surface. If a surface which

is in a state of equilibrium is stretched or compressed the number of adsorbed molecules per unit area is temporarily decreased or increased and consequently the surface tension is increased or reduced until a redistribution of the molecules as a result of diffusion restores the equilibrium.

The time in which this equilibrium is reached in a thin layer of liquid can be calculated by application of the theory of diffusion, which results in the criterion $Dt/L^2=1$ where L is the thickness of the liquid layer (BIRD STEWART and LIGHTFOOT 1960).

Theoretical values of diffusion coefficients D can be derived by applying the formula of Stokes-Einstein $D\mu/T=\text{const}$ or later modifications of this formula. In accordance with experience this formula predicts diffusion coefficients in aqueous solutions at room temperature of the order of 10^{-5} cm²/sec. By using the formula of Stokes-Einstein it is possible to correct experimental values of D for changes in temperature and viscosity μ of the medium

The thickness of the surface lining was approximated as follows. The average total lipoprotein content of a dog's left lower lobe amounted to 160 mg From Fig 4 can be seen about 50 % of the lipoproteins are coming from the peripheral lung regions, say the alveoli This means about 80 mg lipoprotein is originating from the alveoli of the left lower lobe. Fig 6 shows 40 mg lipoprotein diluted in 1 ml Sterofundin has a surface tension of 29 dynes/cm at this dilution viscosity and hence diffusion coefficient do not differ noticable from pure water The total inner surface area of the left lower lobe's alveoli of a dog of about 16 kg will be about 2×10^4 cm² 2 ml lipoprotein solution containing 80 mg lipoprotein spread out on 2×10^4 cm² has a thickness of 10^{-4} cm.

Surface tension in lung alveoli is less than 29 dynes/cm, so the thickness of the surface lining must be less than 10^{-4} cm. However with a thickness of a surface lining of 10^{-4} cm and a diffusion coefficient of 10^{-5} cm²/sec the equilibrium concentration is reached in 10^{-3} sec In other words, thermodynamic equilibrium in the surface lining of lung alveoli occurs within 10^{-3} sec.

5 DISCUSSION

All our experiments were done on living dogs, so they approached reality more than experiments on excised lungs, except for the

experiments with excised lobes which were done in order to compare air P-V diagrams made before and after the removal of surfactant.

The results of our experiments showed the same qualitative alterations of the P-V diagrams when the lobe was filled with fluid as found by most investigators: shifting of the hysteresis loop to the left, narrowing and changing of direction of this loop. According to those investigators these alterations could have been caused by elimination of the surface tension in fluid filled alveoli. As was mentioned shifting of the hysteresis loop to the left was caused by the height of the water column beneath the pressure transducer. If the pressure transducer should have been placed at the level of the dog's sternum it would cause a shifting to the right. This was verified experimentally.

P-V diagrams of fluid filled left lower lobes were calculated from P-V diagrams of air filled lobes of closed-chest dogs, while surface tension was neglected in these calculations.

No calculations were made of air filled lungs of dogs with open thorax.

The small differences between the loops of fluid filled lobes that were calculated and those found experimentally are probably due to the rough assumptions and the neglect of surface tension. If in the alveoli there should not be a substance strongly lowering the surface tension, a higher pressure would be necessary in order to inflate the lung alveoli. In this case we would have found a much greater discrepancy between the calculated and the experimental hysteresis loops.

Therefore the loops of air filled lobes found experimentally are mainly based on visco-elastic properties of the lung and thoracic wall and for about 10 % on surface tension.

In additional experiments with the same but anaesthetized dogs with open thorax lung and thoracic wall compliances were separated. As can be seen higher initial pressures were needed to inflate the collapsed alveoli of these lobes until the lobes volume has reached the volume of the thoracic cavity and the lobe bulges out of the wound.

After this point the ascending curve is nearly identical with the former one. *In vivo* probably most of the alveoli are open because of the existing negative pleural pressure. The pressure difference

between the ascending curves at 50 ml equals about this pleural pressure.

By lung lavage *in vivo* it was also possible to siphon a mixture of lipoproteins which strongly lowered the surface tension of the lavage fluid.

Thus a decrease of surface tension was found from an average of 58 dynes/cm of fresh Sterofundin to 38 dynes/cm of the siphoned lavage fluid.

Figs. 4 and 5 suggest a production of this "surfactant" in the peripheral regions of the lung at a rate of about 1.5 mg/min for the left lower lobe during lavage *in vivo*. When the dog was sacrificed, however, the surface tension of the lavage fluid increased to the average surface tension value of fresh Sterofundin, whereas the lipoprotein concentration decreased to about zero within 20 min after death.

The surfactant, in particular the lipid fraction, might contribute to the hysteresis of the P V diagrams of air-filled lungs. Measurements of lungextracts on the Wilhelmy Langmuir balance lead to this conclusion. On this balance it is possible to measure the surface tension of a solution with varying surface areas caused by a moving trough. When surface tension is recorded simultaneously with surface area during compression and expansion of the surface film of a crude lungextract there is a marked hysteresis. The thickness of the liquid layer in the Wilhelmy Langmuir balance, however, is of a completely different order of magnitude as the surface lining in lung alveoli.

When this layer has a thickness of 1 mm the thermodynamic equilibrium takes place in about 1000 sec. Therefore there will be no thermodynamic equilibrium if the successive surface tension measurements are taken within 1000 sec and so hysteresis will be seen.

In lung alveoli, on the other hand, the surface lining is so thin that thermodynamic equilibrium will occur within one second, so hysteresis of the P V diagrams of air filled lobes cannot be caused by the physico-chemical nature of the surfactant.

It could be argued, however, that the actual concentration of the lipoproteins is much higher than was assumed and that as a result of this liquid becomes more viscous and that consequently the diffusion coefficient becomes smaller. If the concentration is

doubled the thickness of the layer is halved the diffusion coefficient could be four times as small to reach equilibrium in the same time. Therefore the viscosity would have to increase more rapidly than the quadratic of the concentration which is not the case in the concentration range studied.

The experiments with the excised lobes in which practically no difference could be seen between the air P V diagrams of these lobes *before and after the removal of the surfactant, support* the above mentioned theory

The experiments done by MENDENHALL (1963) SUTNICH and SOLOFF (1963) are also in agreement with our theory because they noticed a certain influence of time on their surface tension measurements.

SUMMARY

Unanaesthetized dogs were subjected to the multiple-infusion lavage procedure of the left lower lobe.

Thus it was possible to make *in vivo* static P V diagrams of fluid filled lobes, which were compared with the P V diagrams calculated from *in vivo* static P V diagrams of air filled lobes if these were filled with fluid.

Between the successive compliance measurements surface tension measurements and quantitative *lipoprotein determinations of the siphoned lavage* fluid were made

With these data a contribution of surface tension to lungwork of about 10 % was found, whereas surface lining of the alveol could not have contributed to the hysteresis of lung compliance. Moreover some additional *experiments supported the latter conclusion.*

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SOME OBSERVATIONS ON ANTAGONISM BETWEEN PENICILLIN AND ANTINEOPLASTIC ANTIBIOTICS

BY

A. MANTEN AND J. I. TERRA

1. INTRODUCTION

The chemotherapy of neoplastic diseases is often complicated by severe side effects of the drugs. With the antitumor agents in current use at the moment (alkylating drugs, antimetabolites, antimitotic agents, cytostatic antibiotics, corticosteroids) an adverse effect upon the host-parasite defence mechanism of the patient, such as leucopenia or interference with other natural immunity factors, can but seldom be avoided. To compensate for the side effects of the drugs, antibacterial antibiotics are usually included in the various treatment schedules for neoplastic diseases.

Since many of the cytostatic drugs not only inhibit the proliferation of tumor cells but also display a high antimicrobial activity, especially towards certain bacteria, the question arises whether certain of these drugs may not interfere with the action of antibacterial antibiotics. This possibility is far from being remote, since drug antagonism between the latter antibiotics has often been observed (JAWETZ and GUNNISON 1952, MANTEN and WISSE 1961, MANTEN 1964). It has been demonstrated that especially penicillin G and its semi-synthetic derivatives may be antagonized by some other antibacterial drugs. Therefore it may be assumed that any of the cytostatic drugs may produce the same effect on penicillin.

In the experiments described below we found that this hypothesis is indeed correct. Among other things we observed that even minimum quantities of actinomycin D may strongly counteract the bactericidal action of penicillin G.

2. MATERIALS AND METHODS

The antineoplastic substances used in our experiments were chosen from the various classes of these drugs. As alkylating agents we used high grade commercial preparations of *chlorambucil* (Leukeran (R)) *cyclophosphamide* (Endoxan(R)) and *tri-ethylene thiophosphamide* (Thio-Tepa(R))

From the large group of antimetabolites we investigated *p fluorophenylalanine aminopterin*, *aminopterin*, *amethopterin* (Methotrexate(R)), *5-fluoro-uracil 8-azaguanine* and *6-mercaptopurine* As antineoplastic antibiotics *actinomycin D mitomycin C* and *puromycin* were used. The antibacterial antibiotics used in this investigation were *penicillin* G and *ampicillin* (Penbritin(R))

The above substances were screened for antimicrobial activity by a tube dilution technique employing nutrient broth (Difco) as a medium. The organisms used in these tests were *Streptococcus pyogenes* (Aronson strain) *Staphylococcus aureus* (FDA strain) and laboratory strains of *Escherichia coli* and *Candida albicans*

The technique applied to establish drug antagonism has been described in detail in earlier papers (MANTEN and MEYERMAN WISSE, 1962 MANTEN and TERRA, 1964) In short the tests were carried out as follows nutrient agar plates were homogeneously seeded with relatively dense suspensions of the test organisms and dried. On top of the heavily inoculated plates (approximately 10^8 living bacteria per plate) three paper discs were placed. One disc contained 0.5 U of penicillin G or in other experiments 10 μg of ampicillin (Penbritin(R)), a second one 10 μg of actinomycin D or mitomycin C and the third one the two drugs under test in combination (cf. Figs. 1 and 2) The plates (*primary* plates) were incubated overnight at 37 °C They demonstrated the bacteriostatic effect of the drugs (cf Figs. 1 and 2 on the left) Then replicas were taken by gently pressing velvet stamps of the same inside diameter as the primary plates on these plates, after which transfers were made to fresh plates. Overnight incubation of these revealed the bactericidal effect of the drugs (*replica* plates of Figs 1 and 2 on the right)

The examination of antibiotic antagonism as a function of drug concentration was also carried out employing replica plating Concentrations of penicillin G and of ampicillin corresponding

with approximately 1, 10 and 100 times the minimum inhibitory concentration for the respective test organisms were incorporated in the agar medium and increasing amounts of the second drug (i.e. actinomycin D and mitomycin C) applied in paper discs (cf. Fig. 3).

For the preparation of the discs Whatman filter paper nr. 3 was used, since we found that some other species of filter paper may partly bind actinomycin D.

3. RESULTS

In Table 1 the minimum inhibitory concentrations of the cytostatic drugs are given. It appears from the table that under the circumstances of testing the majority of the substances displayed

TABLE 1

Minimum inhibitory concentrations in µg/ml of cytostatic substances for 4 test organisms. Medium: nutrient broth (Difco) of pH 7.0–7.2. Incubation: 18 hours at 37°C

Substances	*Streptococcus pyogenes* (µg/ml)	*Staphylococcus aureus* (µg/ml)	*Escherichia coli* (µg/ml)	*Candida albicans* (µg/ml)
Alkylating agents				
chlorambucil	>100	>100	>100	>100
cyclophosphamide	50	>100	>100	>100
tri-ethylenethiophosphamide	20	100	>100	100
Antimetabolites				
p. fluorophenylalanine	>100	>100	>100	>100
azaserine	50	>100	>100	>100
aminopterin	2	>100	>100	>100
amethopterin	2	>100	>100	>100
5-fluoro uracil	50	100	100	100
8-azaguanine	100	>100	>100	>100
6-mercaptopurine	100	>100	>100	>100
Antibiotics				
actinomycin D	0.1	0.2	>100	100
mitomycin C	0.2	0.1	5	>100
puromycin	50	>100	>100	>100

only a weak antimicrobial activity For some of the agents even the highest concentration employed (100 µg/ml) did not show any appreciable growth inhibition. That many of the antimetabolites also had only a low order of antibacterial action is probably due to the competition exerted by certain ingredients (amino acids and nucleic acids) of the medium which was used in these tests. Had synthetic or semi-synthetic media of special types been employed, undoubtedly much lower concentrations of the agents would have been found to be growth inhibitory

Among the substances tested actinomycin D and mitomycin C stand out for their very high antibacterial activity As appears from the table concentrations of these antibiotics as low as 0 1 to 0.2 µg per ml may cause a complete bacteriostasis. For this reason, and also in view of the fact that both antibiotics have a potent antitumor activity (KERSTEN and KERSTEN 1963 KAWAMATA, 1963) they were used for further experimentation.

For the investigation of the interaction between actinomycin D and penicillin G three strains of *Staph. aureus* were used (*Staph aureus* FDA, and two patient strains) What was observed is exemplified by the findings recorded in Fig 1

As shown in Fig. 1 on the left, actinomycin D as well as penicillin G and the combination of the two drugs display a pronounced inhibitory action on *Staph. aureus* More interesting however is the replica plate (on the right) showing the bactericidal action of the drugs. This part of the figure shows that penicillin G has killed nearly all the bacteria within its zone, whereas in the zone produced by actinomycin D many bacteria have survived. Apparently the action of penicillin G is bactericidal, whereas that of actinomycin D is much more bacteriostatic. In the zone produced by actinomycin D plus penicillin G also many organisms have survived. It is therefore evident that whereas penicillin G alone is capable of killing nearly all bacteria, its lethal effect is lost when it is used together with actinomycin D The influence of actinomycin D upon penicillin G is therefore antagonistic.

The above results were also obtained with the two other strains of *Staph. aureus*, which emphasizes the general validity of the phenomenon.

A different reaction was observed in experiments on the combined action of mitomycin C and ampicillin (Penbritin(R)). Fig 2 shows

the results of such an experiment using as a test organism *Salmonella typhi murium*

It is evident from Fig 2 that mitomycin C in contrast to actinomycin D (cf Fig 1) possesses a pronounced bactericidal action. Ampicillin on the other hand is in this experiment only partly bactericidal The figure further shows that mitomycin C does not interfere with the (partly) bactericidal action of ampicillin

Essentially the same results were obtained when the above experiment was repeated with test strains of *Salmonella paratyphi B* and of *Escherichia coli* In experiments with the three test strains of *Staph aureus* mentioned earlier in this report we found that the bactericidal action of penicillin G also was unaffected in the presence of mitomycin C

The influence of drug concentration upon the above mentioned antagonism was studied on the same three strains of *Staph. aureus* The results obtained with the combination of penicillin G and actinomycin D acting upon one of these strains is shown in Fig 3.

The picture in Fig 3 shows three rows The figures in the top row indicate the concentrations of penicillin G incorporated into the agar and the amounts in μg of actinomycin D applied per disc The pictures of the petri dishes (primary plates) in the second row show that there is no growth of the test organisms in the presence of 0 05 0 5 and 5 U of penicillin G per ml (1 10 and 100 times the m i.c of *Staph aureus*) Growth occurred only on the control plate on the left The replica plates (bottom row) show the bactericidal action of the two drugs Round the places where the discs containing increasing amounts (1 3 10 and 30 μg) of actinomycin D were applied to the plates containing penicillin G numerous colonies have developed which demonstrates that in the presence of actinomycin D many staphylococci have survived the bactericidal action of penicillin G The interference with the penicillin action appears to be very pronounced at the level of 0 05 U /ml of penicillin G concentration and tends to decrease at higher levels of this antibiotic It is also clear from the picture that low amounts of actinomycin D are more active in counteracting the bactericidal action of penicillin than are the higher quantities of the former substance

The experiment which has been described above was also carried out with the two other strains of *Staph aureus* The results obtained

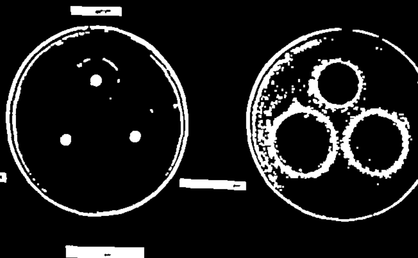


Fig. 1 Antagonism between penicillin G and actinomycin D (for explanation see te

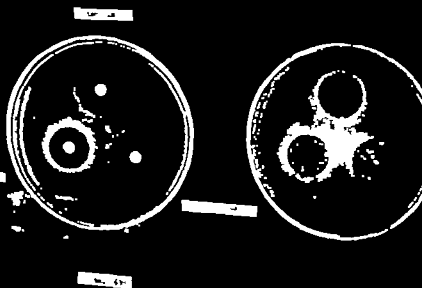


Fig. 2 The single and simultaneous actions of mitomycin C and ampicillin (for explanation see text).

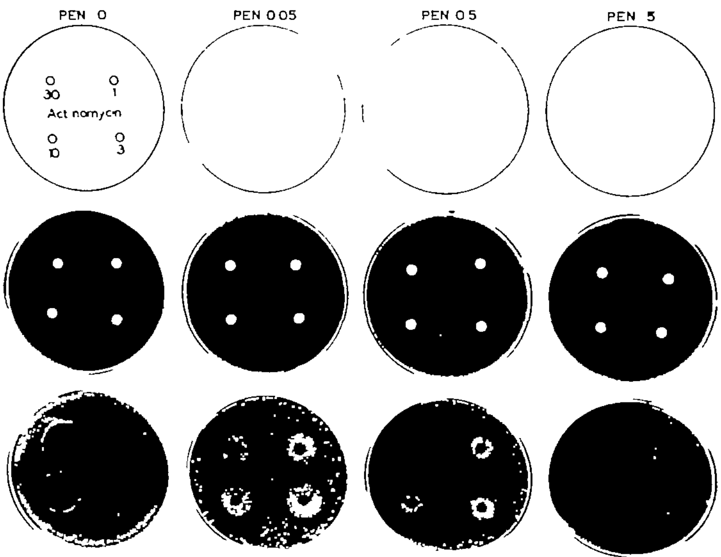


Fig 3 Penicillin antagonism at various concentrations of penicillin G and actinomycin D (f explanat on see text)

TABLE 2

Antagonism between penicill G and actinomycin D as function f drug concentration. Results obtained employing as test organisms three strains of Staph. aureus

Strains	amount of actinomycin D in paper discs (µg)	conc. of penicillin G in agar medium 0.05 U/ml	0.5 U/ml	5 U/ml
Staph. aureus FDA 209	1	+++	+++	–
	5	++	++	–
	10	+	+	–
	20	+	–	–
Staph. aureus 2093	1	+++	+++	++
	5	+++	++	+
	10	++	–	–
	20	–	–	–
Staph. aureus 2236	1	+++	++	++
	5	+++	++	++
	10	+++	++	+
	20	+++	+	–

+++ = strong antagonism
++ = moderate antagonism
+ = weak antagonism
– = no antagonism

with all three strains of this organism are summarized in Table –.

Table * shows that, but for some variations at relatively high penicillin G and actinomycin D concentrations, all strains reacted to various amounts of these drugs in essentially the same way In all strains low to moderate concentrations of penicillin G (0 05 to 0.5 U/ml) appear to be maximally antagonized by minimum amounts (1 µg/disc) of actinomycin D

DISCUSSION

It appears from this investigation that both actinomycin D and mitomycin C possess a pronounced antibacterial action. However there may be a fundamental difference between the two drugs In all test organisms which we used the action of actinomycin

D was biostatic whereas under the *same experimental conditions* mitomycin C invariably displayed a strong biocidal activity If this difference of actions would also apply to tumor cells and there are strong reasons to believe this this observation may contribute to an evaluation of these antibiotics as antineoplastic drugs.

As shown in our experiments actinomycin D may interfere with the bactericidal action of penicillin G The same has been observed with the bacteriostatic antibiotics chloramphenicol tetracycline and erythromycine (JAWETZ and GUNNISON 19..* 1953 MANTEN 1956 MANTEN and TERRA 1964) In any scheme of *antibiotic antagonism actinomycin D might therefore be placed* in one group with the latter antibiotics

The study of the antagonism as a function of the concentration of the antibiotics has shown that low to moderate concentrations *of penicillin G (0 05 to 0 5 U/ml)* were strongly antagonized by *low amounts (1 µg)* of actinomycin D At higher concentrations of penicillin G the antagonism tended to decrease This would imply that if the antagonism would also appear during the clinical use of the drugs prescribing relatively high doses of penicillin G *may partly* compensate for the effect of actinomycin D

Recently actinomycin D and mitomycin C attracted great attention in relation to their modes of anticellular action Though we are aware of the fact that up to now the mechanisms have not been completely elucidated it seems to us that sufficient is known to make an attempt at explaining our findings.

According to KERSTEN (1961) actinomycin D inhibits the DNA dependent synthesis of RNA in cells by specifically binding to the guanine moiety of DNA which finding has been fully substantiated *by later observations (MACH and TATUM 1963* KAWAMATA 1963) As RNA in bacteria as well as in e g mammalian cells has a major function in governing the biosynthesis of proteins actinomycin D ultimately interferes with the production of cellular protoplasm. The substance therefore has a biostatic type of action which is *in accordance* with our observations on the effect of this drug on bacteria The mode of action of mitomycin C on the other hand is probably more complex. This substance has been found to cause *primarily a breakdown of cellular RNA which is followed* by a degradation of DNA due to the liberation of DNA-specific enzymes (KERSTEN and KERSTEN 1963 SUZUKI and KILGORE,

1964) It appears from these investigations that mitomycin C may inflict a heavy damage on cells. That a substance like this has a biocidal type of action, as we have found in certain bacterial species is therefore not surprising

Penicillin G exerts its bactericidal action through an inhibition of the synthesis of the bacterial cell wall or parts thereof, while leaving the production of cytoplasm unaffected (PARK and STROMINGER, 1957 LEDERBERG and ST CLAIR, 1958) If the bacteria are in the actively dividing stage, this necessarily leads to rupture of the bacterial cell membrane and subsequent lysis and death. As was explained above, actinomycin D probably affects the formation of cytoplasm by selectively inhibiting the synthesis of messenger RNA. In other words, in the presence of actinomycin D the bacteria are prevented from producing cytoplasm, which is a "conditio sine qua non" for the bactericidal action of penicillin. Therefore, actinomycin D antagonizes penicillin G

That in our experiments mitomycin C in contrast to actinomycin D did not show any interference with the bactericidal actions of penicillin G and ampicillin, is perhaps due to the multi-site anti cellular action of mitomycin C This substance has been found not only to cause large damage to the DNA RNA system of cells in general but it also may affect – probably as a direct consequence of the damage inflicted upon RNA – the formation of normal cell walls of bacteria. The aberrant morphological forms obtained by exposing certain bacteria to mitomycin (DURHAM 1963) are highly reminiscent of similar forms produced by the penicillins. That the bactericidal action of penicillin G and ampicillin is not antagonized by mitomycin C may possibly be due to their partly similar modes of action.

SUMMARY

The antineoplastic antibiotics actinomycin D and mitomycin C have been investigated by means of replica-plating methods and using some strains of gram negative bacteria. It was found that

1) The antibacterial action of actinomycin D is of the biostatic type whereas mitomycin C ppeared to be biocidal.

2) *In vitro* actinomycin D may strongly counteract the bactericidal action of penicillin G The minimum quantity of actinomycin D employed in our experiments (1 µg) was found t be maximally active in this respect.

3) Mitomycin C did *not antagonize the bactericidal action of penicillin G* and of ampicillin (Penbritin(R))

An attempt is made at explaining the findings by what is known up to now of the mechanism of action of the substances in question.

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VISUAL EVOKED RESPONSES TO BLANK AND TO CHECKERBOARD PATTERNED FLASHES

BY

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J. A. LUBBERS and Th. A. C. SPOOR

1. INTRODUCTION

Since the introduction of averaging methods to extract evoked cortical responses from their noisy background (DAWSON 1954) the response to the presentation of unpatterned (blank) flashes has been studied by several authors and groups of authors. Very little work has, however, so far been done on the response to patterned flashes.

SPEHLMANN (1965) compared various aspects of the response to blank (unpatterned) and to patterned flashes. Blank flashes were obtained by illumination of a blank white square cardboard subtending the central 40–60° of the visual field by a strobe lamp placed behind the subject, who was placed in an otherwise darkened room. Patterned flashes were produced by illumination of cardboards showing various geometrical patterns (line patterns, checker board patterns) in black and white. Flash frequency was between 0.5/sec and 1/sec. Various electrode positions were used; the records presented were obtained from one electrode 3 cm or less to the side of the inion and one at the ipsilateral ear. The EEG was recorded on a 16-channel inkwriter and simultaneously either on tape for off-line analysis by a Mnemotron CAT computer, or directly fed into the latter. In averaging of the potentials in the first 500 msec after the flashes 100–300 responses were summated.

Results are described in terms of surface positive components. Responses to diffuse light were described as having their maximum surface-positive component at 80–120 msec. In some of the 15 subjects, a smaller surface-positive component at 120–200 msec could be distinguished; earlier components at 20–60 msec were

less consistent These components can be tentatively identified with the Γ Z and A elements according to the notation proposed earlier (RIETVELD 1963)

In the case of checkerboard patterned light the maximum surface-positive component had a latency of 180–250 (–375) msec, and its amplitude was larger than that of the largest positive wave in the response to diffuse light It was often preceded by a large surface negative wave with about the same latency as the main surface positive wave in the response to diffuse light in the same subject The characteristic features of the pattern response became the more marked as the number of squares into which the cardboard was subdivided increased from 4 to 16 to 64 to 256, that is as the number of contrast borders and their total length increased. This and the fact that the pattern response reverts to the blank response when the checkerboard pattern is viewed through a +10 D lens, so that the borders become blurred strongly suggests that the pattern response is due to the presence of contrast borders in the stimulus flash The responses to both diffuse and patterned light reached their maximum in an area within 3 cm forward and to the sides of the inion MACKAY *et al* (1960) using largely self-developed apparatus studied responses to variously patterned stimuli. Among the instruments developed are a four-channel averager using a closed loop of magnetic tape as an accumulator and an electronically controlled optical system for the presentation of various combinations and sequences of steadily illuminated patterned fields. Intra individual variations of the responses evoked under seemingly identical conditions and the influence on the response of changes in stimulus properties were studied in one subject over an extended period of time single sessions with 8 subjects were carried out to investigate inter individual variations under identical conditions

Subjects sat in a dentist s chair in a relatively sound proof cubicle and viewed a circular stimulus field at a distance of 100 cm before the eyes they fixated a dim fixation spot in the centre of the field which subtended an angle of about 9 degrees Patterned and unpatterned fields had the same overall luminosity Stimulus fields were presented for one second or more. A grounded electrode was placed at the left earlobe bipolar leads of various kind could be used by leading off from any electrode pair from an array of

electrodes constituted as follows one electrode is placed over the inion and another at the vertex together with these two five additional midline electrodes divide the inion vertex distance in 6 equal parts two additional electrodes are placed at 1/6 inion vertex distance to the right and the left of the inion electrode respectively and two more at 1/6 i-v distance frontal to these electrodes. Averaging is done over 16–*0 responses.

Among the patterns presented were checkerboard patterns, with visual angles subtended at the nodal point by the side of the unit square of 2' 5' 8 1*' and 40' respectively The general features of the pattern responses were similar to those described by Spehlmann in the majority of cases a large positive wave at about 200 msec is preceded by a surface-negative wave at some 100 msec There was no marked increase in amplitude of the main positive wave with increase of fineness of the checkerboard pattern. It increased, however with increase of contrast level. The effect of isolated defocussing was not investigated.

The present is a report on experiments not unlike those of Spehlmann and of MacKay *et al.* in which part of their *conclusions* were confirmed, and their observations were extended in several directions.

2. MATERIALS AND METHODS

In all, 8 healthy young males (Nos. I–VIII) were available as subjects. They all had a visual acuity of *0/*0 so there were no appreciable differences in pattern resolution.

The general features of the pattern responses, as compared to the responses to blank flashes, were studied *in all* eight subjects the influence of unit square size in Nos V–VIII. In one subject (VII) the influence of flash intensity the contribution of the central (fovea, perifovea) and peripheral portions of the *retina* to the pattern response and the location of the cortical response epicenters were studied.

Experiments are made in a dark, soundproof room. Subjects are dark-adapted (> 30 min) their pupils are dilated by instillation, into the conjunctival sac of 10 per cent phenylephrine-HCl. An apparatus described earlier (RIETVELD *et al.*, 1965) is used for the presentation of stimuli. Flash duration is 3 µsec flash intensity is 3 mL unless otherwise noted. When blank flashes are presented

a 50 per cent neutral density filter is interposed so total luminous flux is the same as in the case of patterned flashes. Lengths of unit square sides are tabulated below together with the corresponding angles subtended at the nodal point and the side lengths in the retinal image

Pattern No	side length (mm)	length on retina (μ)	angle subtended
1	35	1060	4 1
2	17 5	530	2° 03
3	13	382	1 35
4	8 75	258	1 0
5	6,87	20.	43 48
6	5	147	34 24
7	4	118	27 30
8	3	88	20′ 36
9	2,2	64	15 10″
10	1,5	44	10′ 15
11	1 35	39	9′ 15
12	1 1	32	7 36
13	0 5	14	3 4

Flash frequency is 2/sec In the main series experiments, the active electrode lies 1½ cm above the inion the reference electrode at the earlobe and the ground electrode at the vertex.

Responses are averaged over two series of one hundred flashes, with a 5-sec pause between the series. Three dry series are run before the actual experiment Potentials are recorded on a Grass Polygraph in addition the output of the polygraph amplifying system is fed into a Nuclear Data Enhancetron computer or stored frequency modulated on tape for off line averaging

3 RESULTS

3 1 General features of response

Fig 1 shows left the response to blank flashes for each of the subjects the corresponding responses to the presentation of pattern No 6 (5 × 5 mm² unit squares) are shown on the right. The blank responses have the characteristics described earlier (Rietveld 1963) a more or less well-developed negative peak at about 60 msec (B) is followed by a positive trough at some 100 msec (Γ)

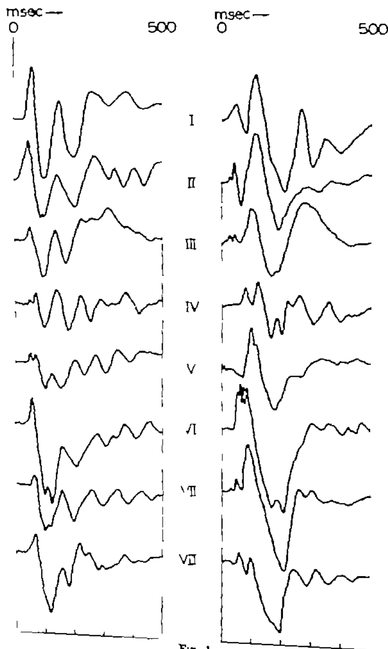


Fig. 1
Blank responses (left) and responses to checkerboard patterns (right) of the subject I VIII.

subsequent waves (*E* surface-negative latency in the 1[illegible]0–210 msec range and *Z* surface-positive at some 200–235 msec) are more or less clearly marked.

In the pattern response *B* appears in nearly all cases to be somewhat smaller than it is in the response to blank flashes; the main differences from the blank responses however are an inversion of *Γ* (large surface *negative* peak at the same latency as that of *Γ* in the blank response) and a marked deepening of *Z*.

For two of the subjects (Nos. I and VII) the blank responses (top) and the pattern responses (bottom) are shown in Fig. 2, to allow a closer comparison of the response types.

This behaviour was found in seven of the eight subjects, the exception being No. VI, who also differs from the others in that the *B* wave is replaced by a number of fast oscillations.[1] His

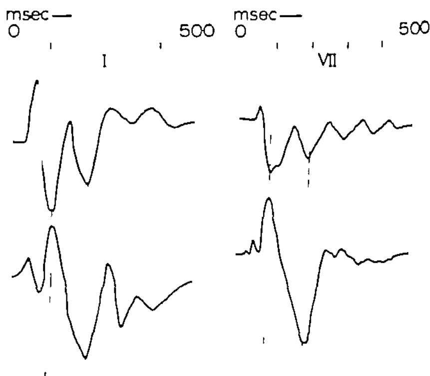


Fig. 2.
Blank response (top) and pattern response (bottom) of subjects I and VII.

[1]) The appearance, *in this subject*, of fast oscillations at 50–80 msec upon presentation of blank flashes was remarked on earlier (RIETVELD 1965, where he was numbered Subject II).

responses will be analysed in a separate paper As in the experiments of Spehlmann, blurring causes the response to revert to the blank type.

3.2. INFLUENCE OF UNIT SQUARE SIZE

Fig 3 (subject VII) shows the response to blank flashes, as recorded at the beginning of the session (0) the responses to patterns 1-13 and the blank flash response recorded at the end of the session. With pattern No. 1 Γ is less deep than in the blank response it becomes progressively smaller upon presentation of patterns and 3 Inversion occurs between patterns 3 and 4 the inverted (negative) Γ wave reaches its maximum at patterns 7 and 8 it then becomes smaller until a polarity reversal back to surface-positivity occurs between patterns 11 and 12. With patterns 1 to 3 Z is smaller than in the blank flash response it then deepens progressively to reach maximum depth at pattern 8 then it becomes smaller until at pattern 12 it is again less deep than in the response to unpatterned flashes. In Fig 4 the values, with reference to zero of Γ and Z amplitudes are plotted against unit square side length normalized absolute values with reference to a common zero are given in Fig. 5 The influences of unit square size on Γ and Z amplitudes are strikingly similar as unit square side length decreases from 35 mm (pattern 1) to 3 mm where the angle subtended is about 60 min of arc, amplitudes rise at an increasing rate a further diminution of unit square size results in a sharp drop in amplitude As a result the response amplitude as measured between Γ and Z depends on unit square size in the way pictured in Fig 6 which shows the $\Gamma-Z$ amplitude after subtraction of the corresponding amplitude in the blank response In three of the four subjects tested the response amplitude had its maximum upon presentation of pattern 8 in one response was maximal when pattern 10 (angle subtended about 10 minutes of arc) was presented.

3.3 INFLUENCE OF FLASH INTENSITY

The influence of flash intensity on the pattern response was investigated in subject VII. Pattern No 10 (unit square size 1.5×1.5 mm^2) was presented at low (0 5 mL) standard (3 0 mL) and high (10 mL) flash intensity Results are pictured in Fig. 7

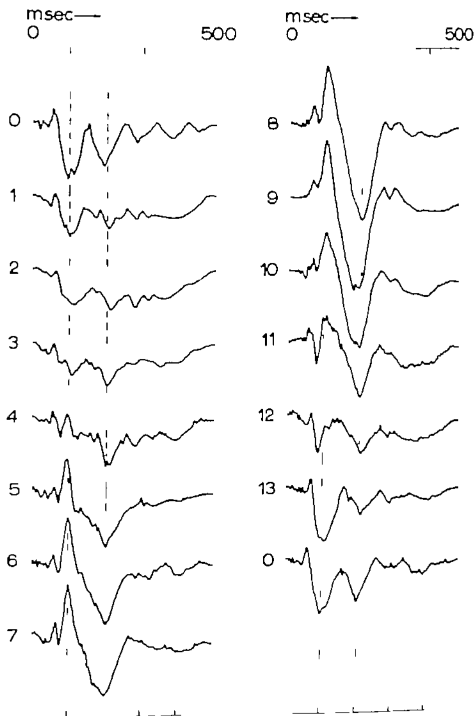


Fig 3

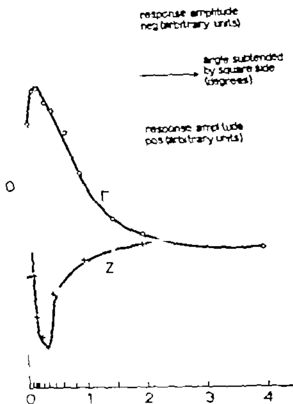


Fig. 4.
Subject VII. Blank response (0) and response to each of the patterns 1 t 12

At low and medium intensity Γ inversion occurs between patterns 3 and 4 at high intensity it is already present at pattern _. For all intensities, reversal to surface-positivity occurs at pattern 0. The deepening of Z is more marked at medium and high intensity than at low intensity In some cases (for instance at medium intensity pattern 10) the inverted Γ wave mixes with E this results in a change in shape the peak becoming more rounded, and in an apparent change in implicit time. The same is found with coarse patterns at high intensity Fig 6 shows the $\Gamma - Z$ amplitude after subtraction of the amplitude in the blank response for the three flash intensities.

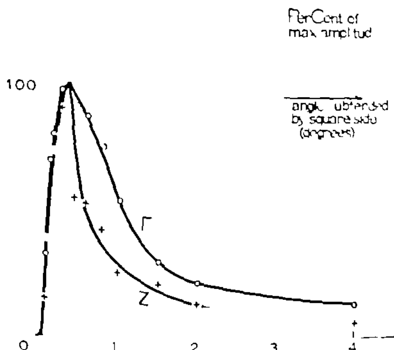


Fig. 5.
Cf Fig. 4 Normalized absolute values of Γ and Z amplitude with reference to a common zero.

3 4 Contribution of central and peripheral areas of retina

The contributions of the central and the peripheral areas of the retina were studied in two ways ... by presentation of patterns consisting of central black discs of increasing diameter surrounded by a checkerboard pattern (No 8) and by presentation of checker board patterns of increasing area The results of an experiment of the first type are given in Fig 9 which shows from top to bottom the full pattern response and the responses when the angles subtended by the black central discs were .. .. 54 and 4 04 respectively Γ–Z amplitude is down to about 60 per cent when the black disc subtends 2 degrees and to about 30 per cent when the angle subtended is 2 54 at 4 0 4 no vestige of the pattern response remains. Fig 10 shows left the blank responses, and right the pattern responses (pattern No 8 standard intensity) for angles subtended by the patterned area as indicated. For the

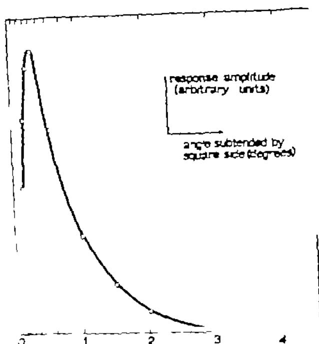


Fig 8.

$\Gamma - Z$ amplitude as function of unit square side length.

pattern response recordings, vertical gain is half that for the blank response recordings. Both the blank response and the pattern responses are virtually fully developed when the angle subtended by the diameter of the patterned area is 4 degrees.

This is clearly apparent from Fig 11 where the amplitude as measured between the inverted Γ wave and the Z wave is plotted, after subtraction of the amplitude at the corresponding latencies, in the blank response against screen area.

The results of these experiments are in complete agreement both indicate that the central foveal area contributes by far the greatest part to the pattern response

The way in which the latency of the principal elements in the pattern response depends on screen size is pictured in Fig 12. Screen size does not influence the implicit times of B and Z that of the inverted Γ wave increases sharply as screen size drops below 5 sq cm.

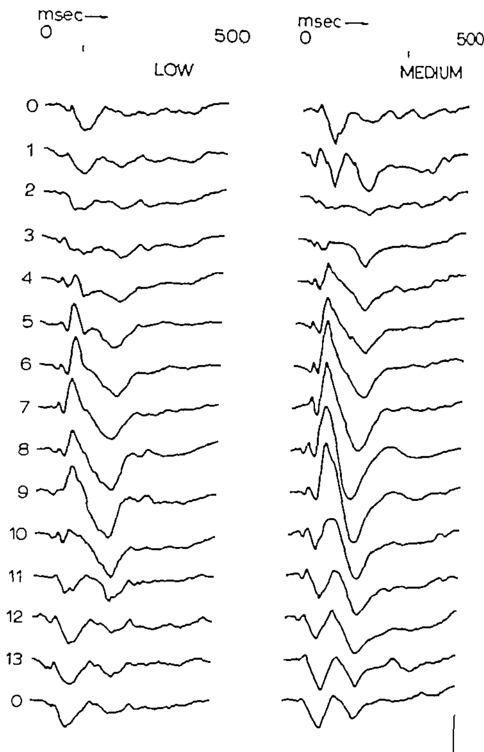


Fig. 7a
Influence of flash intensity on pattern response

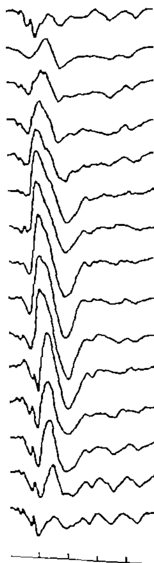


Fig 7b
Influence of flash intensity on pattern response.

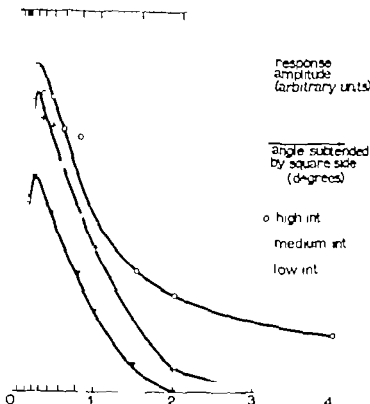


Fig. 8.
Γ–Z amplitude after subtraction of that in the blank response, plotted against unit square side length, for different flash intensities.

3.5 Localization of pattern response

Fig. 13 shows the responses to presentation of checkerboard pattern No. 8 as led off between a reference electrode at the right earlobe and each of eight active electrodes. The hindmost of the midline electrodes (no. 1) is at 1.5 cm before the inion; the other midline electrodes (nos. 2, 3 and 4) lie at 4, 8 and 12 cm before no. 1 respectively. Electrodes 5 and 6 lie on either side of no. 1 at a distance of 1.5 cm from the midline, nos. 7 and 8 on either side of no. 2 at a distance of 4 cm.

Inversion of Γ is restricted to the leads from nos. 1, 2, 5 and 6, that is, to a small area in the paramedian occipital region. Z deepens progressively as we pass from no. 4 through no. 3 to no. 2. At nos. 5 and 6 it is clearly less deep than at nos. 1 and 2; at nos. 7 and 8 it is not much deeper than at no. 4. It would seem that the

pattern effect on Z (deepening) extends over a somewhat larger area than that on Γ (inversion)

Records from bipolar leads between the electrode pairs indicated are shown in the left-hand portion of Fig 14 (left blank responses right pattern responses) Electrodes are on a circle with a radius of cm, centered on a point 3 cm forward from, and 2.5 cm to the side of the inion. Dashed verticals in the records are at ~100 msec (Γ) and at ~175 msec (Z). The right-hand portion of Fig 14 shows the direction of the equivalent dipoles at these latencies (arrows from positive to negative) At 100 msec, the midline positivity in the case of the blank response changes into midline negativity upon presentation of patterned flashes at 175 msec,

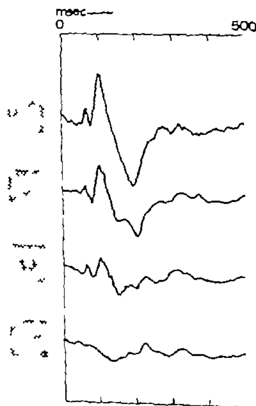


Fig. 9.
Contribution of central foveal area to pattern response.

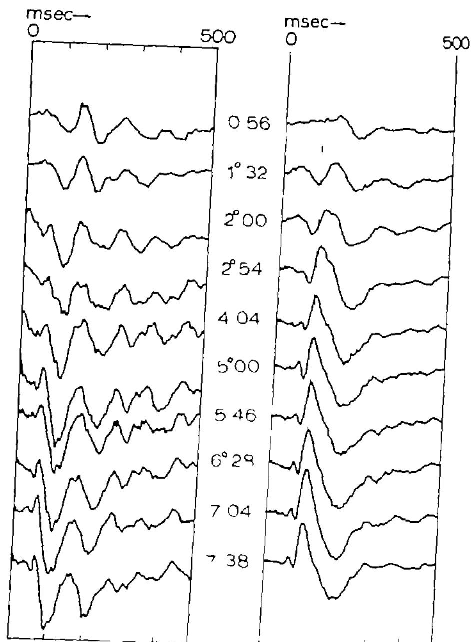


Fig 10
Blank responses (left) and pattern responses (right) f r angles subtended by screen area indicated

the change in direction of the electrical axis is much less marked. This is in agreement with the fact that Z unlike Γ is not inverted.

§ 6. Determinants of pattern response

If we change from a coarse checkerboard pattern to a finer one the total number of contrast borders as well as their total (combined) lengths increase. The way in which the total number of contrast borders, and their combined length depend on the number of squares in which a square checkerboard with a side equal to unity is subdivided is shown in Table 1 where l stands for side length of the squares N for their number n for the number of contrast borders, and L for their combined length.

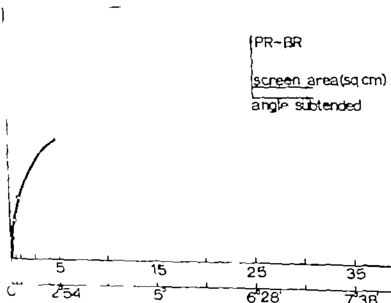


Fig. 11

Cf. Fig. 10. Pattern response minus blank response as function of screen area.

In an attempt to see which of the two number of contrast borders or their combined length, is the more important for the generation of the pattern response, the response to a checkerboard pattern was compared to that evoked by stripe patterns consisting

of parallel black and white stripes of equal width and where either the number of contrast borders or their combined length was the same as in the checkerboard patterns. As is apparent from Fig 15 the response to the stripe patterns resembles the

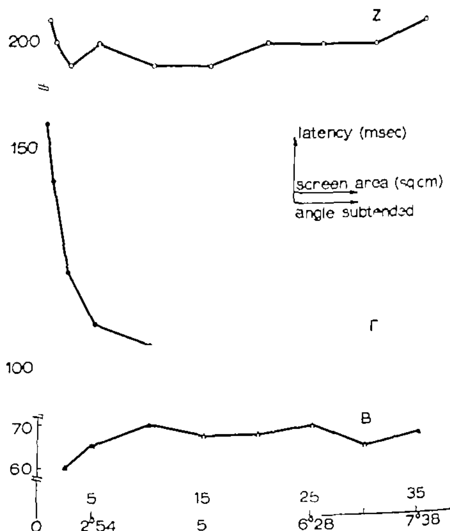


Fig 12.

Latencies of B inverted Γ and Z as a function of pattern screen area.

TABLE I

I	N	n	L
2^{-4}	2^{10}	2^{5}	[illegible]
2^{-6}	2^{4}	2^{6}	2^{5}
2^{-3}	2^{6}	2	2^{4}
2^{-4}	2^{4}	2^{6}	2^{4}

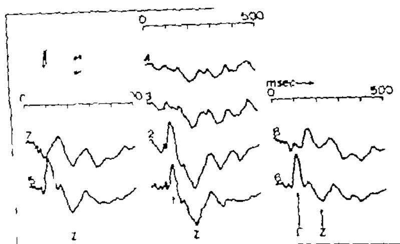


Fig. 15.
Unipolar responses from electrodes placed as indicated.

blank response rather than the response to presentation of a checkerboard pattern. This strongly suggests that for the generation of the pattern response the presence in the pattern, of intersecting contrast borders is essential. The question then naturally arises whether changes in the angles of intersection lead to changes in the response.

As a preliminary to experiments where, among other things the response to presentation of diamond patterns was studied, it was ascertained that the *orientation, in the frontal plane*, of the checkerboard pattern is immaterial the *response* does not change when the pattern is rotated through 360° This means that there are no preferred directions, as might complicate the analysis of skew patterns. Fig 16 shows the response to a checkerboard

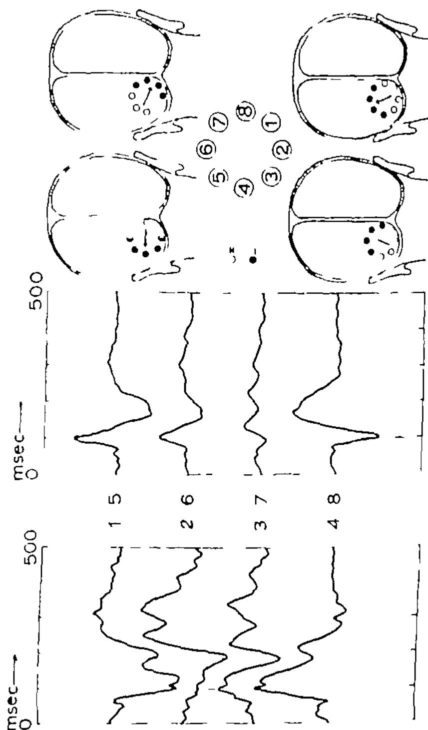


Fig 14

Blank responses (left) and pattern responses (right) a led off bipolarly between the electrode pairs indicated

pattern, and to patterns of diamonds having very nearly the same unit area, and acute angles of 60° 45 and 30° respectively Clearly the diamond response resembles the checkerboard response but its amplitude is markedly less and diminishes as the acute angles become smaller

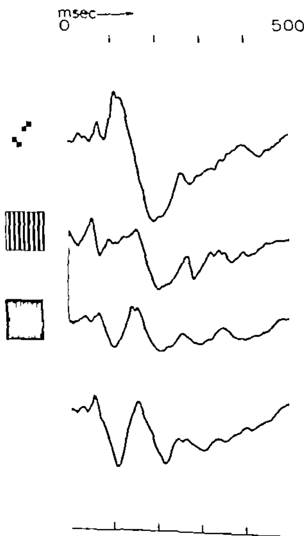


Fig 16.
Responses t checkerboard pattern, stripe patterns, and t blank flashes.

Starting from a given checkerboard pattern we can construct three diamond patterns derived from it: in one the area of the unit diamonds *is equal to the unit square area; in the second, side length, and in the third,* height remains the same All three types were used: for a given value of the diamonds angles, responses to all three were about equal.

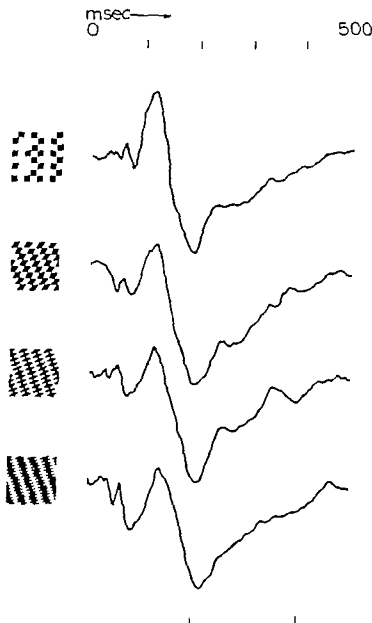


Fig 16

Responses to checkerboard pattern and to diamond patterns of increasing skewness.

If for a given value of the angles, the diamonds are replaced by parallelograms the amplitude of the response becomes the smaller as the difference between the lengths of adjoining sides increases, until it reverts to the blank response, while the subject experiences the pattern as a stripe pattern rather than as a pattern of parallelograms. The response to presentation of a pattern of isolateral triangles (unit area again being "equal" to unit square area) was, to all intents and purposes, equal to that to the checker board patterns and, thus much larger than the response to a pattern of diamonds with angles of 60° and 120°. The main difference between these two patterns is, of course the absence of obtuse angles in the triangle pattern. A pattern of isosceles rightangled triangles, finally evokes a response which is even larger than that to the corresponding checkerboard pattern. Taken together these facts strongly suggest that the presence in the pattern, of right or acute angles between contrast borders, is essential and that, in the case of diamond patterns, a positive effect, on the pattern response, of the acute angles becoming smaller is more than outweighed by a negative one due to the concomitant increase of the obtuse angles.

4. DISCUSSION

4.1 GENERAL NATURE OF RESPONSES TO CHECKERBOARD PATTERNS

The difference between responses to presentation of a checker board pattern and to blank flashes appears to be of a quantitative rather than a qualitative nature that is upon presentation of a pattern, elements present in the blank flash response may change in amplitude (*E*) sign (*I*) and latency (*I*) but no new elements appear

It is rather difficult to compare the results obtained in the present experiments with those of Spehlmann and of MacKay e.a. because of the differences in technique The range of unit square sizes used in the present series is larger than in either of the other investigations. In MacKay's experiments, angles subtended by unit square sides ranged from 40 to 2 min of arc, that is, from somewhere between patterns 5 and 6 in the present series to just beyond pattern 13 From the data given it is not possible to calculate the angles subtended in Spehlmann's experiments, but his patterns were comparatively coarse. His results are in agreement with

those found with coarse patterns, in the present series Γ is not yet inverted and response amplitudes still increase with increasing fineness of the patterns. MacKay on the other hand reports a surface – negative wave at some 100 msec, which might well be the inverted Γ as found with fine patterns in the present experiments the large surface – positive wave at some 200 msec could tentatively be identified with the deepened Z-wave in our experiments He finds no marked influence of fineness of pattern on the amplitude of this wave The range of unit square lengths used by MacKay *et al* (2 to 40) includes the length (15 to 20') where in the present experiments amplitude passes through its maximum. This might well explain why no increase in amplitude with increase of pattern fineness was reported. The amplitude increase with increase of contrast level as reported by MacKay c.s. was also found upon changing from 0 5 mL to 3 0 mL flash intensity in the present experiments.

The region where in the present experiments the cortical epicenter of the pattern response was located is well within the area in which according to Spehlmann the response maximum is located.

The rotation by more than 180 of the equivalent dipole at 100 msec when instead of blank flashes patterned flashes are *presented is in agreement with the fact that the* (inverted) Γ wave has its origin far back For a possible explanation of the inversion a displacement of maximum activity to more superficial layers might be invoked (also cf ANOKHIN 1964)

4 2 THE RELATION BETWEEN UNIT SQUARE SIDE LENGTH AND RESPONSE AMPLITUDE

The way in which response amplitude depends on the side length of the unit squares composing a checkerboard pattern (cf Section 3 2) deserves consideration This holds in particular for the sharp drop in amplitude as the angle subtended by side length drops below some 20 minutes of arc and the length of its retinal image below some 100 μ [1]) Limitation of resolution cannot

1) It is of interest to note that in completely different experiments (*stimulation with sinusoidally modulated light*) SPEKREIJSE (1966) finds a similar dependence of amplitude on square size

very well be invoked. Under continuous illumination all subjects had *0/*0 visual acuity under stroboscopic illumination as in the experiments, it decreased to 1 /20 which is still sufficient to perceive subtending 2 min. Subjectively the pattern was, in the case of the finer patterns, perceived as a system of lines rather than as an array of squares.

It appears more probable that this behaviour indicates the presence, in the system, of neuronal connexions subserving interactions over retinal distances of about 100 μ Of course it is not necessary for such connexions to lie in the retina itself indeed, the amount of processing done in the periphery is much less in mammals than in fishes and amphibians. Such an interaction would then have a spatial extent which is over a fairly wide range of illumination levels, independent of flash intensity As stated in Section 3 4 the pattern responses are to all intents and purposes

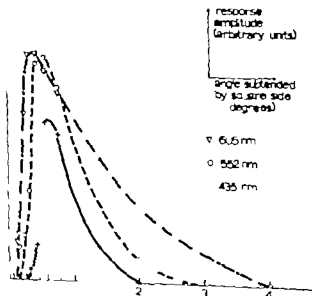


Fig 17

Pattern response amplitudes, as function of unit square side length, for red, green, and blue flashes.

generated in the central foveal area alone that is, in an area where only cones are present. The question arises whether the supposed interaction would extend to neuronal elements connected with different cone types or be restricted to those connected with one type only. Fig. 17 shows the pattern response amplitudes as a function of the angle subtended by the side of the unit squares for red (605 nm), green (552 nm) and blue (435 nm) flashes. The curve for red flashes practically coincides with that for white flashes. It will be seen that the maxima do not coincide. This *suggests that interactions do not extend from one system to* another; the position of the maxima suggests that the spatial extent of the interaction is greater for the blue system than for the others.

SUMMARY

The cortical response to the presentation of patterned flashes was compared with the response evoked by blank flashes. Subjects were dark-adapted, and their pupils were dilated. Flash duration was 3 μsec; flashes were presented at */sec. In the black and white patterns the combined area of black units was equal to that of the white ones. Total luminous flux was the same in the case of patterned flashes and of blank flashes. In the main series experiments, unipolar leads were used (active electrode 1½ cm above the inion, reference electrode at one earlobe, ground electrode over vertex). Responses were averaged over a series of 100 flashes each, with the aid of a Nuclear Data Enhancetron computer.

The principal results were the following:

1. The fully-developed response to presentation of a checkerboard pattern is characterized by an inversion of Γ and a marked deepening of E;
2. The degree of development of the pattern response depends on unit square size; as patterns change from coarse (side length in image on retina about 1000 μ) to fine, $\Gamma-E$ amplitude increases to reach a maximum at an image length of some 100 μ; a further increase in fineness of the pattern causes a precipitate drop in response amplitude;
3. The position of the amplitude maximum is, over a fairly wide range of intensities, not dependent on flash intensity;
4. Virtually all of the pattern response is generated in the central foveal area
5. The response to a stripe pattern closely resembles that to a blank flash; this means that the presence of intersecting contrast borders is essential;
6. Diamond patterns elicit a pattern response the magnitude of which is the less as the acute angles in the pattern are sharper; the response to a pattern of equilateral triangles is equal to that evoked by a checkerboard pattern of the same unit area; that to a pattern of isosceles right

angled triangles is larger that that evoked by presentation of a checker board pattern of equal unit size area.

It is concluded that the pattern response is probably due t spatial interaction between neuronal elements, extending over a distance corresponding with some 100 μ on the retina.

Experiments with coloured patterned flashes indicate that probably such an interaction does not take place between elements belonging t different cone systems.

ACKNOWLEDGEMENT

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THE INFLUENCE OF ALERTNESS ON THE AMPLITUDE OF THE VISUAL EVOKED RESPONSE AND ON REACTION TIME

BY

W. J. RIETVELD, W. E. M. TORDOIR AND J. R. B. HAGENOUW

1 INTRODUCTION

In recent years several authors have studied the effect of attentiveness, induced by the presentation of a warning signal preceding a test stimulus, on the reaction time (*RT*) when the subject is instructed to respond in a prescribed way to the test stimulus as fast as possible. Most of them agree that there is a range of time intervals between the warning signal and the test signal where the presentation of the former diminishes the reaction time, the degree of diminution depending on the actual value of the interval. Whether the warning signal is in the same modality as the test signal is immaterial. The reduction in *RT* is supposed to be due to a shortening of central processing time.

The alerted state is characterized, among other things, by some degree of inhibition or suppression of α activity; this would suggest some kind of correlation between diminution of *RT* and α blocking.

On the other hand, attempts have been made to find correlations between the reaction times on the one hand and amplitude and latency of cortical response elements on the other. The opinion seems to be rather generally held that short reaction times go hand in hand with larger amplitudes and with smaller latencies; also attentiveness is held to increase response amplitude. It has been shown, however, that in vigilance tasks where the subject is in the attentive state the amplitude of the cortical evoked response to a given stimulus may be either larger or smaller than in the no task situation, depending on whether the task is easy

or difficult. Also the degree of α blocking in the attentive state depends on things like the intensity of the test stimuli and the degree of habituation of the subject to the procedure This means that the whole matter of the relation between attentiveness, cortical evoked response α blocking and RT needs to be reconsidered.

LANSING *et al* (1959) studied reaction times and α-blocking in alerted and non-alerted conditions. Test stimuli were 10 μsec light flashes subjects were instructed to press a reaction key in response to the stimuli RT's were measured with a Beckman electronic counter activated by 1000/sec pulses the counter started at the moment of the flash and stopped when the reaction key was pressed. Stimuli were presented at intervals varying from 15 to 45 sec. The alerted state was brought about by a buzzer signal preceding the test stimulus by an interval varying from 50–1000 msec the various intervals being applied in random sequence

In the non-alerted state the mean reaction time was 280 msec, no matter whether at the time of the stimulus α-activity was well developed or partly or completely suppressed.

For buzz flash intervals from 50 to 250 msec, α-activity was incompletely blocked and mean RT was 255 msec in the interval range 300–1000 msec, α blocking was complete and RT fell to 200 msec

The authors describe a progressive reduction of RT from its 280 msec value in the unalerted state as the click-flash interval increases from 50 to 300 msec. A further increase of interval duration has little effect on RT The authors point out that the percentage of trials in which complete α-blocking occurs increases from zero at a 50 msec interval to one hundred at an interval of 300 msec These facts suggest that α-blocking and reaction time are governed by a common process which might well be activation of the reticular system Why in the unalerted state RT is independent of the level of α-activity at the moment of stimulation remains unclear It is also unclear why the interval range was not extended upward to include at least one value where RT has passed its minimum.

DONCHIN and LINDSLEY (1966) studied reaction times to 10 msec flashes and the corresponding evoked responses, as averaged

by means of a Mnemotron CAT computer A warning click was given in all cases in part of the series the subject was specially alerted by what is referred to as a feedback condition he was told what his *RT* was and urged to shorten it.

Click flash intervals of either 1 1½ 2 or 2½ sec were applied in random sequence The meaning of this is unclear since no further reference to the intervals is made and results are not grouped according to interval duration the only effect of using varying intervals would seem to be the unnecessary introduction of one more uncontrolled variable In general short *RT*'s were found to be associated with large amplitudes feedback shortened *RT*'s and increased amplitudes However in a no-feedback series immediately following two feedback series, *RT* was long but amplitude remained about the same as in the preceding series. The explanation given for this dissociation appears rather weak the previous set encouraging the subject to compete with himself is invoked. Why this set would continue to affect amplitude rather than *RT* itself is not clear

No warning signal was given in the experiments of MORRELL and MORRELL (1966) who studied the variability of response element latencies and amplitudes and of reaction times under unvarying conditions stimuli were (weak) 10 msec flashes given aperiodically at intervals ranging from 8–14 sec. No statement is made as to whether they were given in random sequence or not. Data were processed with the aid of a LINC computer Here again shorter reaction times were found to be associated with larger response amplitudes there was no consistent relation between response element latencies and reaction times. Experiments by RIETVELD *et al* (1966) showed that attentiveness is not necessarily linked to large response amplitudes in the case of both auditive and visual vigilance tasks the amplitude is smaller when the task is difficult and larger when it is easy On the other hand the degree of α blocking was found not to depend on the attention level alone stimulus intensity as well as habituation exert an influence on the degree of suppression of α-activity

These findings suggest that the picture according to which there is a simple association between alertness on the one hand, and α blocking large response amplitude and short *RT* on the other is oversimplified

The present is a report on experiments where the dependence of RT's, evoked response amplitudes, and α blocking on the duration of the interval between warning signal (click) and test signal (flash) was studied.

2. MATERIAL AND METHODS

Five normal subjects were used in these experiments; the experimental set-up was not unlike that of Lansing o.a.

Experiments were done in a dark, soundproof, electrically shielded room. The subject is seated in front of a 20 × 20 cm² opal glass window, placed at 30 cm before his eyes, and onto which flashes delivered by a G. R. Strobotac can be projected. Screen luminance is about 50 mL. This low value was chosen to minimize artefacts. The pupils are dilated by instillation into the conjunctival sac, of 10 per cent phenylephrine-HCl. During the experiments, the subject's eyes are open; a piece of plain white paper is placed at a few cm before the eyes. The flasher is placed in a soundproof box so that its clicks are inaudible. Test stimuli are offered at intervals varying randomly between 5 and 10 sec. Prior to the flash, a 2 msec warning click is given, an electric pulse being fed into an earphone worn by the subject. Click flash intervals are 0, 50, 100, 200, 300, 500 and 1000 msec; the instruction is to press a reaction key which stops a Beckman electronic counter that started at the moment the flash was offered.

One experimental session consists of 5 series, in each of which all seven intervals are used 20 times in random order, so 140 RT's are determined in each series, while, in one session, RT for each interval is measured 100 times.

3. RESULTS

3.1 REACTION AS A FUNCTION OF CLICK FLASH INTERVAL

Reaction times of one subject (means and standard errors of the means) are plotted against click flash interval in Fig. 1a, which also shows the average reaction time as found in series where flashes only were offered at intervals randomly varying between 5 and 10 sec.

It will be seen that in the interval range from zero to and

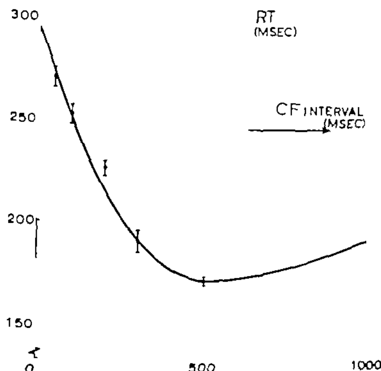


Fig. 1a.
Linear plot of reaction time against C–F interval (one subject).

including 100 msec *RT*'s exceed those in the non alerted state. Over the 0–500 msec interval range *RT* decreases regularly with increase of interval duration from the high of 290 msec at zero interval to a minimum of 170 msec at an interval of 500 msec; a further interval decrease results in an increase in *RT* which at a one-second interval is about 185 msec (*RT* is back at 290 msec after some 10 sec).

The relation between *RT* and interval is virtually the same for all five subjects, as is apparent from Fig. 1b, where *RT*'s in per cent of that at zero interval are plotted against interval duration.

3.2 Cortical response amplitude as a function of click flash interval

Fig. 2 shows the averaged evoked potentials as recorded from the occipital area of the subject to which Fig. 1a refers. For each of the intervals 85 responses were averaged.

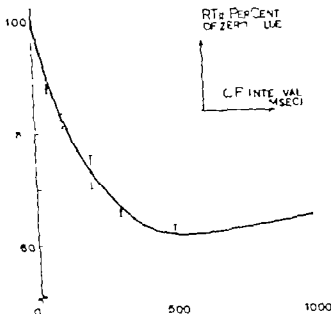


Fig. 1b.
Normalized linear plot of RT vs C–F interval (all five subjects).

In all cases a surface-negative wave at about 60 msec after the flash is followed by two surface-positive waves, at about 100 and 200 msec, respectively. These waves can be tentatively identified as *B*, *Γ* and *Z* following the notation proposed earlier (RIETVELD 1963). The response proper – which has a duration not exceeding 300 msec – is followed by marked after-oscillations, whose amplitude does not seem to be systematically dependent on click flash interval. The latencies of recognizable response elements are not influenced by the duration of the interval.

Response amplitude as measured between *B* and *Z* is plotted against click flash interval in Fig. 2a (amplitudes in arbitrary units). Clearly there is no monotonous relation between interval duration and response amplitude, which has a maximum for the 200-300 interval range. Normalized amplitude *vs.* interval plots for all five subjects are given in Fig. 2b. Though the behaviour is far less regular then in the case of the *RT*'s, as witness the large standard errors of the means, the maximum at a 300 msec interval appears to be well established.

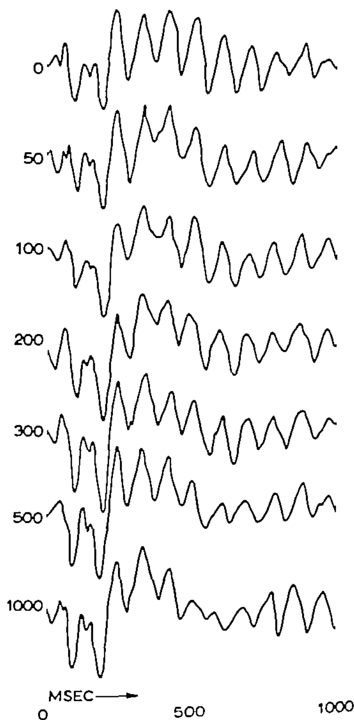


Fig 2.
Occipital evoked potential for each of the O F intervals (subject to which Fig 1a refers)

In view f the low frequency of stimulation, the question has to be considered whether the measured $B-Z$ amplitude is influenced by artifacts. Simultaneous recordings from occipital and frontal leads are shown in Fig. 4 (click-flash interval 0 msec). Though the Z-wave in the occipital recording does not coincide in time with the deep surface-positive trough in the frontal potential, a change in amplitude of the latter might affect that of the occipital Z-wave. If, however frontal and occipital recordings at zero interval are compared with those at an interval value of 300 msec, it ppears that the increase in occipital response amplitude at 300 msec is accompanied by decrease in amplitude of the frontal potential (Fig 5). This warrants the tentative conclusion that disturbing influences of the frontal artifact are of minor importance.

If the warning click should evoke a response in the occipital area this might, at low interval values, become superimposed on the flash-evoked potential (Cigánek, 1966).

Averaged occipital potentials following simultaneous presentation of click and flash (zero interval) are shown, together with those following the presentation of the flash only and of the click only in Fig 6. In the occipital lead hardly any effect of the click is t be discerned, while the response to the flash only is, if anything, even somewhat greater than that to the click and flash simultaneously It follows that the flash-evoked potential is not contaminated by the response to the click.

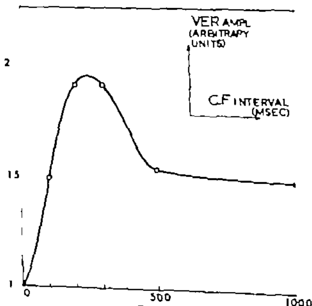


Fig. 3a.
Linear plot of VER amplitude against C-F interval (same subject in Fig 1).

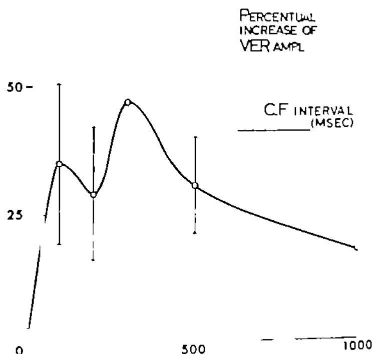


Fig 3b
Normalized linear plot of VER amplitudes against C–F interval (all five subjects)

3 3 ALPHA BLOCKING AS A FUNCTION OF CLICK FLASH INTERVAL

Alpha blocking following the warning signal could be studied in three of the five subjects, the other two showing no appreciable α activity in the relaxed state There is no suppression of α-activity at interval values up to 100 msec in the 100–1000 msec interval range the percentage of cases where α activity is suppressed at the moment of the flash increases with interval duration as is apparent from Fig 7 where this percentage is plotted against interval length At an interval of 500 msec α activity is suppressed in 80 per cent of the trials at 1000 msec it is still present in 10 per cent In the subject to whom Fig 1 refers response amplitudes and reaction times in a series where the background EEG was desynchronized were compared to amplitudes and *RT*'s in a series where α activity was present at the moment of the flash As is apparent from Fig 8 the amplitude was larger in the non-desynchronized state Reaction time on the other hand was the same (105 msec during synchronization as against 107 msec during α blocking)

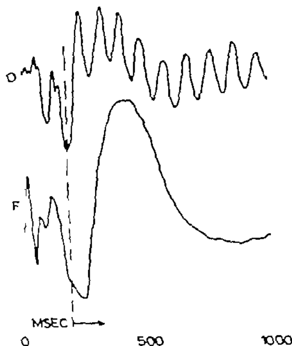


Fig. 4.
Simultaneous recordings from occipital (O) and frontal (F) leads (C-F interval 0 msec)

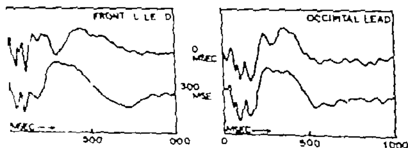


Fig 5
Simultaneous recordings from occipital and frontal leads (C-F intervals 0 and 300 msec).

4 DISCUSSION

The main conclusion to be drawn from the results obtained in the present series of experiments is that the relation between attentiveness α blocking evoked response amplitude and reaction time is more complicated than is usually assumed. More specifically *RT* and response amplitude values are not monotonically related to the percentage of trials in which at the moment the test stimulus was delivered α blocking was suppressed in fact amplitude passes through a maximum at an α blocking percentage of about 55 (300 msec interval) and *RT* goes through a minimum at 80 per cent α blocking (500 msec interval) As to the relation between α blocking percentage and reaction times our findings are somewhat at variance with those described by LANSING c.s. though inspection of their figure 2 reveals some similarities on points which were not mentioned or not commented on in their text The description of their figure 2 runs as follows "There is shown in Fig 2 the relaxed *RT* level at 280 msec The alerted *RT* curve shows a progressive reduction below this level as the preperiod of warning increases from 50 to 300 msec with periods longer than 300–400 msec

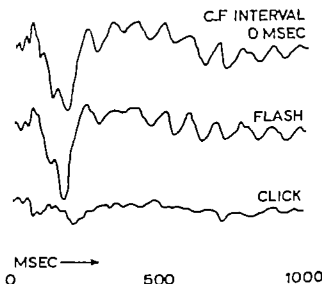


Fig 6

Occipital evoked potential to simultaneous click and flash, flash only and click only

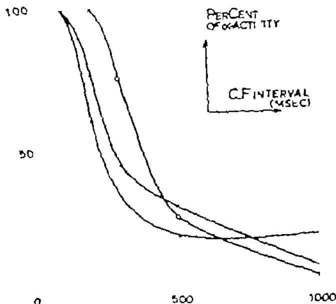


Fig. 7
Alpha-blocking as a function of C-F interval

very little further reduction in *RT* occurs" The figure itself shows, however that at a 50 msec interval, *RT* exceeds that in the relaxed state by some 25 msec, and that at 100 msec it is down against to the relaxed' value. Also there is an indication that the *RT* passes through a minimum at an interval value of some 600 msec, after which it tends to increase again. These facts are in good agreement with the present findings The α blocking vs interval duration curve given by Lansing c.s. has an inverted-sigmoid shape similar to that found in the present investigation, the main difference being that, in their case there would already seem to be some small incidence of α blocking at the 180 msec interval value and that in their case blocking is already found in some 90 per cent of the cases at 400 msec. This percentage is reached, in the present experiments at a 1-second interval only also the minimum is far deeper in the present case

The fact that for interval values of 100 msec and less, *RT* in the alerted state exceeds that in the relaxed state needs further clarification. It may be of significance in this respect that in the

4 DISCUSSION

The main conclusion to be drawn from the results obtained in the present series of experiments is that the relation between attentiveness α blocking evoked response amplitude and reaction time is more complicated than is usually assumed. More specifically *RT* and response amplitude values are not monotonically related to the percentage of trials in which at the moment the test stimulus was delivered α blocking was suppressed in fact, amplitude passes through a maximum at an α blocking percentage of about 55 (300 msec interval) and *RT* goes through a minimum at 80 per cent α blocking (500 msec interval) As to the relation between α blocking percentage and reaction times our findings are somewhat at variance with those described by LANSING e.a though inspection of their figure 2 reveals some similarities on points which were not mentioned or not commented on in their text The description of their figure 2 runs as follows 'There is shown in Fig 2 the relaxed *RT* level at 280 msec The alerted *RT* curve shows a progressive reduction below this level as the preperiod of warning increases from 50 to 300 msec with periods longer than 300–400 msec

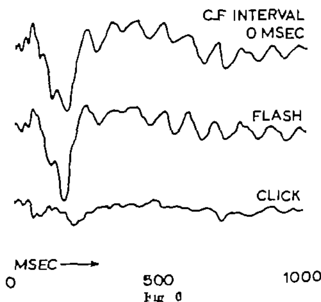


Fig 6

Occipital evoked potentials to simultaneous click and flash flash only and click only

by α blocking which is absent in the 0–100 msec range nor is there any feature in the first-named curve to account for the fact that, at intervals of 200 msec and over amplitude decreases with increase of interval

It follows that, in the case of response amplitudes also α blocking is not the sole determinant, and that other so far unknown, factors must be operative

SUMMARY

In five subjects the way in which the amplitude of the cortical responses to presentation of light flashes, and the corresponding reaction times depend on the length of the interval between a warning click given prior to the test stimulus and the flash itself was studied, together with the incidence of α-blocking at the moment the flash was given.

With intervals below 100 msec RT is longer than in the non alerted state; in the 0–400 msec range it decreases with increase of click flash interval; it slowly increases again as the interval is further increased. The amplitude of the cortical evoked response shows a sharp increase with increase of interval from zero to about 200 msec, to diminish again upon further lengthening of the interval. Response latencies are not systematically dependent on interval duration. Alpha-blocking does not occur at interval lengths below 100 msec; from 100 msec up its occurrence increases with increase of interval duration until, at an interval of one second, it is present in 90 per cent of the trials.

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0–100 msec interval range there is no α blocking while for intervals up to 500 msec, RT diminishes as the α blocking percentage increases This might suggest that, at least in the range of short click flash intervals, another factor tending to lengthen RT is operative which is in the region where α blocking occurs overridden by the postulated RT shortening influence of α suppression Some kind of interference between sensory channels might well be involved The question then arises whether a similar increase of RT at low interval values over the relaxed value would also be found if warning stimulus and test stimulus were in the same modality Experiments where the warning signal as well as the test signal are visual stimuli and where retinal interaction is excluded and interaction at geniculate level is minimized are in preparation So far no explanation can be offered for the fact that *at interval values over 500 msec RT (slowly) increases with increase of interval* while α blocking further increases

Comparison of the α blocking *vs* interval and response amplitude *vs* interval curves shows that the marked and fast increase in amplitude in the 0–200 msec interval range is not solely occasioned

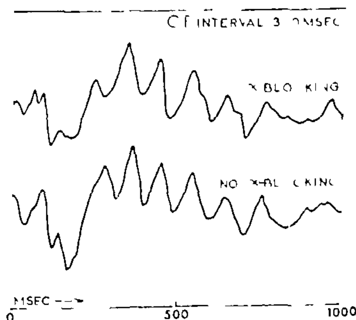


Fig 8

Occipital evoked potentials in a period of desynchronization and in the presence of alpha-activity

by α-blocking which is absent in the 0–100 msec range nor is there any feature in the first-named curve to account for the fact that at intervals of 500 msec and over amplitude decreases with increase of interval

It follows that in the case of response amplitudes also α blocking is not the sole determinant, and that other so far unknown, factors must be operative

SUMMARY

In five subjects the way in which the amplitude of the cortical responses to presentation of light flashes, and the corresponding reaction times depend on the length of the interval between a warning click given prior to the test stimulus and the flash itself was studied, together with the incidence of α-blocking at the moment the flash was given.

With intervals below 100 msec RT is longer than in the non-alerted state; in the 0–500 msec range it decreases with increase of click-flash interval; it slowly increases again as the interval is further increased. The amplitude of the cortical evoked response shows a sharp increase with increase of interval from zero to about 200 msec, to diminish again upon further lengthening of the interval. Response latencies are not systematically dependent on interval duration. Alpha-blocking does not occur at interval lengths below 100 msec from 100 msec up its occurrence increases with increase of interval duration until, at an interval of one second, it is present in 80 per cent of the trials.

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TIME COURSE OF RESPONSE OF THE MEMBRANE-COVERED OXYGEN ELECTRODE

BY

A. BERKENBOSCH

1. INTRODUCTION

In previous papers (BERKENBOSCH and RIEDSTRA 1963a, 1963b) the effect of various factors influencing the steady state response in amperometric oxygen determinations with the membrane-covered Clark electrode was considered in some detail. Among other things, expressions were derived for the value of the diffusion current and for the temperature dependence of sensitivity. In the present the non-steady-state case, i.e. the time course of the response, will be investigated.

2. THEORY

In the following the case of linear diffusion in a direction normal to a plane source will be considered. Diffusion coefficients are assumed to be independent of concentration.

Let in Fig. 1 the left hand compartment represent the membrane (membrane thickness x_1, solubility coefficient of oxygen in the membrane S_1, diffusion coefficient of oxygen in the membrane

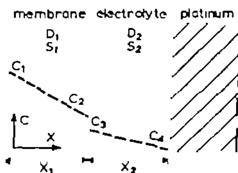


Fig. 1

D_1) while the right-hand compartment represents the electrolyte (x_2, S_2, D_2). The product, S_1 D_1, is the membrane permeability P_1; similarly S_2 D_2 can be called the 'permeability' of the electrolyte.

Let the free surface of the membrane be in contact with a medium of constant pO_2, p and let c_1, c_2, c_3, c_4 represent oxygen concentrations.

In each of the compartments the rate of diffusion before the attainment of the steady state is governed by Fick's second law

$$dc/dt = D(\partial^2 c/\partial x^2)$$

Though this equation has been solved, for a variety of initial conditions and boundary conditions, for the one-layer system, no general solution is known. In the case of the membrane-covered electrode the one-layer system is approximated if the contribution to the response of the electrolyte layer is negligible.

I One layer system

Initial and boundary conditions are

$$c = c_1 \text{ for } x = 0$$
$$c_1 = 0 \text{ for } t = 0$$
$$c = c_2 \text{ for } x = x_1$$
$$c_2 = 0$$
$$0 \quad x < x_1$$

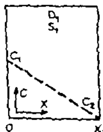


Fig. 2.

According to Barrer (1951) the total amount of oxygen diffusing during the time t through a unit area of the membrane is given by

$$Q = x_1 c_1 \left[\frac{D_1 t}{x_1^2} - \frac{1}{6} - \frac{2}{\pi^2}\sum_{1}^{\infty}\frac{(-1)^n}{n^2}\exp\left(-\frac{D_1 n^2 \pi^2 t}{x_1^2}\right)\right] \qquad (1)$$

where $r = 1, 2, 3$

Eq. (1) can be written as

$$Q = x_1 c_1 \left[\left(\frac{D_1 t}{x_1^2} - \frac{1}{6} \right) - \left\{ \frac{2}{\pi^2} \sum_1^\infty \frac{(-1)^r}{r^2} \exp\left(- \frac{D_1 r^2 \pi^2 t}{x_1^2} \right) \right\} \right] =$$

$$= x_1 c_1 \left(\frac{D_1 t}{x_1^2} - \frac{1}{6} \right) - \frac{2 x_1 c_1}{\pi^2} \sum_1^\infty \frac{(-1)^r}{r^2} \exp\left(- \frac{D_1 r^2 \pi^2 t}{x_1^2} \right)$$

and since $c_1 = S_1 p$ and $D_1 S_1 = P_1$ so $c_1 = p(P_1/D_1)$

$$Q = p \frac{P_1}{x_1} \left[\left(t - \frac{x_1^2}{6 D_1} \right) - \frac{2 x_1^2}{\pi^2 D_1} \sum_1^\infty \frac{(-1)^r}{r^2} \exp\left(- \frac{D_1 r^2 \pi^2 t}{x_1^2} \right) \right] \tag{1a}$$

The term $(t - (x_1^2/6D_1))$ is negative, zero or positive according as

$$t < \frac{x_1^2}{6D_1}, \quad t = \frac{x_1^2}{6D_1} \quad \text{or } t > \frac{x_1^2}{6D_1}$$

The quotient $x_1^2/6D_1$ which in the following will be written as τ can be used as a measure of the time lag of the establishment of the steady state. It can be called the characteristic time of the response. The use of τ was introduced in a study on heat conduction by JAEGER (1950).

For time $\to \infty$ the sum of the exponentials approaches zero so

$$Q_{t\to\infty} = p \; \frac{P_1}{x_1} (t - \tau)$$

and if $\varrho_1 = x_1/P_1$ is the resistance to diffusion through the membrane

$$Q_{t\to\infty} = \frac{p}{\varrho_1} (t - \tau) \tag{2}$$

At any time $t > 0$ the diffusion current I_t is given by $I_t = n \cdot F \cdot q$ where n is the number of Faradays required per mole of electrode reaction, $F = 96500$ Coulombs and q is the oxygen transfer in moles per second. For the present case where four electrons are involved in the processes occurring at the electrode this gives

$$I_t = \frac{386000}{22400} q^* = 17.232 \, q^*$$

where q^* is oxygen transfer in ml per second and since

$$q^* = a \; (\mathrm{d}Q/\mathrm{d}t)$$

where a is membrane area,

$$I_t = 17.232\, a \, \frac{dQ}{dt} \tag{3}$$

It follows from Eqs. (2) and (3) that, for large t

$$Q_t = \frac{1}{17.23\ a} \int_0^t I_t dt$$

so that,

$$17.232 \frac{ap}{\beta l}(t-\tau) = \int_0^t I_t dt$$

and

$$\tau = \frac{I_s t - \int_0^t I_t dt}{I} \tag{3a}$$

where I is the steady-state value of I at time $\to \infty$

It follows that in a plot like that of Fig. 3 where (I_t/I) 100 is plotted against time the ordinate erected at time τ intersects the response vs time curve at the point for which the shaded areas are equal.

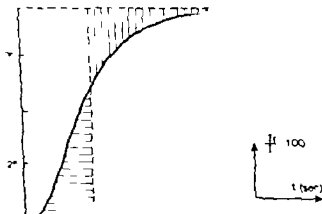


Fig. 3.
Response curve of an electrode, as delivered by the recorder. At $t = 0$ pO_2 is abruptly changed from zero to 490 mm Hg. Membrane 37.3 μ Teflon FEP. Temperature 25 C.

It follows from Eq (1a) that for $t = \tau$

$$Q = \frac{p}{\varrho_1}\left[- 2\frac{x_1}{\pi^2 D_1}\sum_1^\infty \frac{(-1)^r}{r^2} \exp\left(- \frac{r^2\pi^2}{6}\right)\right] \tag{4}$$

Values for $\frac{(-1)^r}{r^2} \exp\left(- \frac{r^2\pi^2}{6}\right)$ are

for $r = 1$ $-0\ 193$
$r = 2$ $+0\ 0003$
$r = 3$ $-4\ 13 \times 10^{-6}$

It follows that if for $t > \tau$ we neglect all but the first term the Q value is still correct to within a small fraction of one percent For $t > \tau$ Eq (1a) can then be simplified to

$$Q_t = p\left[\frac{(t-\tau)}{\varrho_1} + 2\frac{x_1 S_1}{\pi^2} \exp\left(- \frac{D_1\pi^2 t}{x_1^2}\right)\right] \tag{5}$$

From (3) and (5) it follows that for $t > \tau$

$$I_t = 17\ 232 a\ \frac{p}{\varrho_1}\left[1 - 2 \exp\left(- \frac{D_1\pi^2 t}{x_1^2}\right)\right] \tag{6}$$

and for the steady state response at time $\rightarrow \infty$

$$I_s = 17\ 232 a\ \frac{p}{\varrho_1} = 17\ 23\ a\ p\ \frac{P_1}{x_1} \tag{7}$$

The sensitivity σ i e the steady-state diffusion current at unit oxygen partial pressure thus equals $\sigma = 17\ 32 a\ (P_1/x_1)$ From

$$I_t = I_s\left[1 - \ \exp\left(- \frac{D_1\pi^2 t}{x_1^2}\right)\right] \tag{8}$$

we have

$$\frac{I_s - I_t}{I_s} = 2 \exp\left(- \frac{D_1\pi^2 t}{x_1^2}\right) \quad \text{or}$$

$$\log\frac{I_s - I_t}{I_s} = 0\ 301 - \frac{D_1\pi^2 t}{2\ 303\, x_1^2} = 0\ 301 - 4\ 86\frac{D_1}{x_1^2}\ t \tag{9}$$

It follows that for $t > \tau$ a semilog plot of $(I - I_t)/I$ against time will yield a straight line with a negative slope of $4\ 286(D_1/x_1^2)$ and which when produced has its y–intercept at log

For $t = \tau$ Eq (8) becomes

$$I = I_\infty \left[1 - 2 \exp\left(-\frac{\pi^2}{6}\right)\right] = I_\infty (1 - 0.386) = 0.614\, I_\infty$$

so the instantaneous value of I at the time τ = 61 4 percent of the steady-state value It is also easy to calculate the values of t at which the response is 90 95 99 percent of I_∞. A few values are collected in Table 1

TABLE 1

t	(I_t/I_∞) 100
	61 4
1.83τ	90
2.24	95
3.22	99
4.63τ	99 9

.2 TWO-LAYER SYSTEM

This case obtains when the influence of the electrolyte layer is not negligible In those arrangements where a medium such as cellophane or filter paper is interposed the system is four-layered (membrane-electrolyte-medium-electrolyte) but the influence of the electrolyte is very small compared to that of the membrane and the interposed medium, so that the system approximates a two-layer arrangement. For a multi-layer system consisting of n layers with resistances ρ_1 ρ_2 and characteristic times τ_1, τ_2, the value of Q will, for large values of t approach

$$Q = p \; (\rho_1 + \rho_2 + \quad \rho_n)^{-1} \; (t - T^*)$$

where T^* is the characteristic time of the response of the system.

Strictly speaking, this expression holds only in the case where partition rat is equal unity so there are no concentration jumps at the interfaces. This will in general not be so. This can be taken into account by appropriate modifications of the values, and thus of the ρ values.

JAEGER (1950) has developed a method to calculate T^* For a two layer system the expression derived by Jaeger can be written as

$$T^* = \frac{\rho_1 (\tau_1 + 3\tau_2) + \rho_2(\tau_2 + 3\tau_1)}{\rho_1 + \rho_2} \tag{10}$$

It is to be noted that in the case of a two layer system I_{T^*}/I_s will in general not be equal to 0.014.

In the steady state the contribution of the electrolyte to the diffusion current is negligible if $\varrho_1 \gg \varrho_2$; this need not necessarily be the case before the steady state is attained.

For $\varrho_1 \gg \varrho_2$ Eq. (10) can be simplified to

$$T^* = \tau_1 + 3\tau_2 + \frac{\varrho}{\varrho_1}(\tau + 3\tau_1)$$

and since $3 \gg \varrho_2/\varrho_1$

$$T^* = \tau_1\left(1 + 3\frac{\varrho_2}{\varrho_1}\right) + 3\tau_2 \qquad (11)$$

For electrolyte layers of a thickness of a few μ, as is the case when the membrane lies tightly against the electrode, τ_2 does not exceed one or two milliseconds, so in general $\tau_1 \gg \tau$.

For $\varrho_1 \gg \varrho_2$, $\tau_1 \gg \tau_2$ the effect of the electrolyte layer can be neglected in the non-steady state also.

2.3 Influence of temperature on response

The influence of temperature on the diffusion coefficient is given by

$$D_T = D_0 \exp\left(-\frac{E_D}{RT}\right) \qquad (1\ )$$

where E_D is the activation energy of the diffusion process. Since $\tau = x_1^2/6D_1$ we thus have

$$\tau_T = \frac{x_1^2}{6D_{1_0}} \exp\left(\frac{F_D}{RT}\right)$$

so

$$\log \tau_T = \log \tau_0 + \frac{F_D}{2.303RT}$$

where $\tau_0 = \dfrac{x_1^2}{6D_{1_0}}$ and

$$\log \tau_T = \log \tau_0 + 218.64\ F_D/T \qquad (13)$$

A semilog plot of τ against the reciprocal of absolute temperature will for a one-layer system yield a straight line with a positive

slope of 218.64 E_D. The same applies to any value of $t > \tau$ at which a given fraction of the full response is attained.

In the case of a two-layer system there is no simple relation between τ and temperature. With increase of temperature the diffusion coefficient increases, solubility in the electrolyte diminishes, while that in the membrane may either increase or decrease depending on the membrane material. Generally τ will decrease with increase of temperature.

3 MEASUREMENTS

3.1 Methods

Measurements were made with circular platinum electrodes. Standard electrode diameter was 1.5 mm. In a separate series of experiments, electrodes of varying diameter were used to see at which minimum diameter of the electrode the diffusion process can for a given membrane thickness still be considered as being linear. The reference electrode was of the Ag type. Teflon FEP membranes (Du Pont) of various thicknesses were used.

The electrode was pressed against a piece of membrane of a surface area of 3.70 cm² which had been weighed beforehand so its average thickness could be calculated from its weight, area and density. The membrane was not stretched.

The thickness of the electrolyte layer was minimal (a few microns).

Step changes in the pO_2 of the gas flowing along the electrode could be made with the aid of the "gas gun" pictured in Fig. 4, in which the electrode was mounted. The outflow orifice a, of the plunger b which delivers an O_2-N_2 mixture of known composition can abruptly be moved from position 1, where no oxygen flows along the electrode, to position 2, where the gas mixture flowing along the electrode has a known oxygen partial pressure, with the aid of the spring c and the lever d. This takes about 2 msec as measured with the aid of a small metal plate mounted on the protruding part of the plunger, a beam of light and a phototransistor. In position 1 the beam of light falls on the phototransistor, the output of which is displayed on a CRO screen. In position 2 the plate interrupts the beam and output is zero. When the plunger moves from position 1 into position 2, phototransistor output has fallen to 5 percent of its full value within 2 msec.

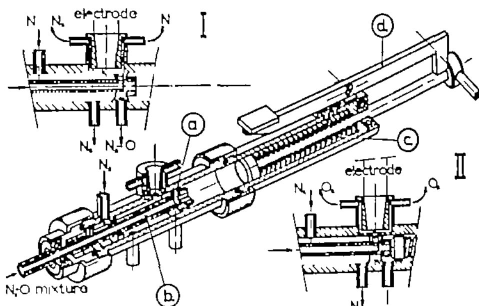


Fig 4
"Gas gun For explanation see text

The gases (flow 150 ml/min) are passed through humidifiers filled with a saturated NaCl solution relative humidity was 75 percent so condensation on the membrane of water vapour upon cooling was prevented

Humidifiers gas gun and electrodes were immersed in a thermostated water bath Temperatures were constant within 0 05 °C either way Standard measurements were carried out at 5 °C Diffusion currents were measured with the aid of a Honeywell 153 X 16 ½-second recorder with full-scale deflection at 10 mV The response curve shown in Fig 3 was delivered by the recorder

According to ROGERS (1964) the permeability of Teflon FEP at 25 C is

$$(P_1 =)\ 500 \times 10^{-12}\ \frac{\text{ml (STP) cm}}{\text{cm}^2\ \text{sec}\ \text{cm Hg}}$$

For a membrane thickness x_1 of about 50 μ as used in the majority of experiments the resistance to diffusion

$$\varrho_1 = x_1/P_1 \sim 10^{+7}$$

For a ϱ_2 of about $10^4 - 10^5$ as is the case when the membrane is pressed tightly against the electrode $\varrho_1 \gg \varrho_2$ so in the steady

state the electrolyte layer can be neglected, and the system can be considered as a one-layer system.

From measured values of I_s, P_1 can then be calculated using Eq (7), while D_1 can be found from $D_1 = x_1^2/6\tau$. The solubility coefficient, S_1 equals P_1/D_1. Activation energies can be computed from the temperature dependence of P and D.

3. Results

3.1 *Time course of response: calculation of permeability and of diffusion coefficient*

A semilog plot of $(I - I_t)/I$ against time for a Teflon FEP membrane of a thickness of 57.3 μ at 25 °C, is shown in Fig. 5

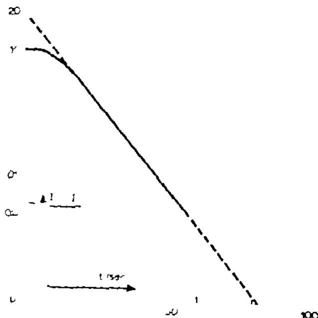


Fig. 5.
(cf. Fig. 3). Semilog plot of $(I - I_t)/I$ against time.

At time zero pO_2 in the gas flowing along the electrode was abruptly changed from zero to 490 mm Hg which resulted in a steady-state diffusion current of 1.58 μA.

The plot shows in agreement with Eq. (9) that for $t > \tau$ a

straight line with a negative slope which when produced has its y-intercept at log 2 is obtained

Fig 5 shows that the diffusion current attains 61 4 percent of its steady state value in 25 6 sec. As marked in Section τ_2 is about once $T^* > \tau_2$ Together with the fact that $\rho_1 \approx \rho_2$ this means that Eq (11) changes to $T \sim \tau$ so here again the influence of the electrolyte layer is negligible

Putting the numerical values for I_s a x_1 and p into Eq (7) we obtain

$$I_s = 1\ 58 \times 10^{-4} = 17\ 232 \times 1\ 767 \times 10 \quad \frac{P_1}{57\ 3 \times 10^{-4}} \ 40$$

so

$$P_1 = 608 \times 10^{-13} \ \frac{\text{ml (STP) cm}}{\text{cm}^2 \text{ sec cm Hg}}$$

which value is in good agreement with that given by Rogers. From $\tau = x_1{}^2/6D_1$ it follows that for τ 25 6 sec $x_1 = 57\ 3 \times 10^{-4}$ cm,

$$D_1 = \frac{x_1{}^2}{6\tau} = 21\ 4 \times 10^{-8}\ (\text{cm}^2\ \text{sec}^{-1})$$

and

$$S_1 = \frac{P_1}{D_1} = \frac{608 \times 10^{-13}}{21\ 4 \times 10^{-8}} = 28\ 3 \times 10^{-4}\ (\text{ml/cm}^3\ \text{cm Hg})$$

3 2 2 *Influence of temperature calculation of activation energies*

Fig 6 is a semilog plot of $(I - I_t)/I$ against time for a Teflon FEP membrane of a thickness of 55 3 μ and for the temperatures indicated Again the produced straight lines obtained for t values $> \tau$ intersect the zero ordinate at log

An alternative way of computing D_1 at any given temperature is to calculate it from the negative slope of the corresponding straight lines using Eq (9).

Figs 7 and 8 are respectively semilog plots of the diffusion coefficient and of the 90 percent response time (1 8τ cf Table I) against the reciprocal of absolute temperature Either can be used to compute the activation energy of the diffusion process by making use of Eqs (12) and (13) respectively
Using Eq (13) we have

$$1740 \text{ sec K} = 2186 4\ E_D \quad \text{so}\ E_D \quad 8\ 0 \text{ kcal/mole}$$

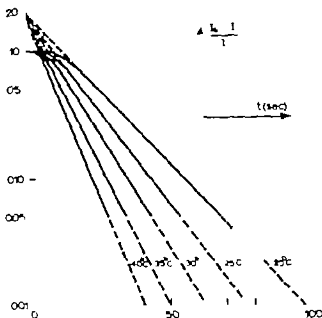


Fig. 6.
Semilog plot of $(I - I_t)/I$ against time.
Membrane 85.2 μ Teflon FEP

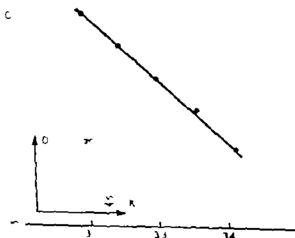


Fig. 7
Semilog plot of the diffusion coefficient against the reciprocal of the absolute temperature

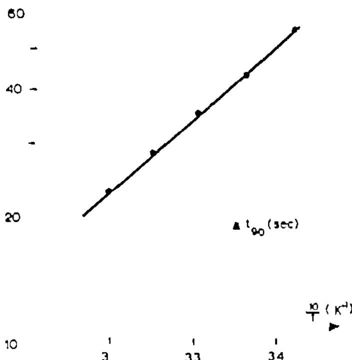


Fig 8.
Semilog plot of the 90 percent response time against the reciprocal of the absolute temperature.

Besides D_1 S_1 also obeys the Arrhenius relationship according to

$$S_{1_T} = S_{1_0} \exp \frac{-\Delta H}{RT} \tag{14}$$

where ΔH is the differential heat of solution From Eqs. (12) and (14)

$$P_{1_T} = P_{1_0} \exp \frac{-E_P}{RT}$$

where $E_P = \Delta H + F_D$

E_P can be found from sensitivity measurements at different temperatures and ΔH by subtracting F_D from F_P

Results of measurements on Teflon FEP types 200 A and 100 A (thickness 2 and 1 milli inch respectively) are collected in Table 2. Values for P and E_P are in good agreement with those given by Rogers (590×10^{-12} and 5.8 respectively at 25 C). It is worthy to mention that at 25 C the solubility of oxygen in Teflon FEP is about 7 to 8 times that in water and that as is apparent from

TABLE 2

25 °C

μ	σ nA/mm Hg	sec	P picoliter (STP) mm/cm sec cm Hg	D μ²/sec	S μl (STP)/cm³ cm Hg	E kcal/mole	E_D kcal/mole	ΔH kcal/mole
60.6	2.99	29.2	5.96	21.0	2.84	5.7	8.2	−2.5
57.7	3.05	28.8	5.78	20.7	2.79	5.7	8.2	−2.5
59.7	2.97	30.0	5.83	19.8	2.94	5.6	7.9	−2.3
56.6	3.20	26.3	5.96	20.3	2.94	5.9	8.0	−2.1
59.3	3.03	29.2	5.94	20.0	2.97	6.0	8.3	−2.3
57.8	3.13	26.2	5.92	21.1	2.81	5.6	8.3	−2.7
58.7	3.13	26.0	6.03	22.1	2.73	5.7	8.2	−2.5
57.5	3.06	26.7	6.82	20.8	2.83	5.6	8.1	−2.5
57.2	3.22	25.6	6.06	21.7	2.79	5.7	7.8	−2.1
55.3	3.30	24.1	6.01	21.0	2.86	5.7	8.0	−2.2
58.3	2.83	30.1	5.42	18.8	2.89	5.8	8.5	−2.7
average			5.89	20.6	2.85	5.7	8.1	−2.4
27.1	6.63	5.93	5.91	20.5	2.88	5.4	7.5	−2.1
25.1	7.23	4.97	5.90	21.1	2.84	5.7	7.8	−2.1
23.9	7.83	4.72	6.15	20.3	3.04	5.9	7.8	−1.9
24.6	7.38	5.09	5.97	19.8	3.01	5.6	8.2	−2.6
25.6	6.43	6.62	6.04	20.6	2.93	5.8	7.5	−1.7
24.2	7.10	5.20	5.65	18.8	3.01	5.9	8.4	−2.5
26.9	6.63	5.91	5.86	20.4	2.87	5.7	8.1	−2.4
27.4	6.69	6.20	6.02	20.2	2.98	5.5	8.2	−2.7
29.5	6.20	7.00	5.91	19.2	3.06	6.1	7.9	−1.8
average			5.94	20.0	2.96	5.7	7.9	−2.2
overall average			5.91	20.4	2.90	5.7	8.0	−2.3

the fact that ΔH is negative it diminishes with increase of temperature. As the absolute value of the positive temperature coefficient of the diffusion coefficient exceeds that of the negative temperature coefficient of the solubility coefficient the temperature coefficient of P is positive but less than that of the diffusion coefficient. As a result of this, the influence of temperature on τ is relatively stronger than that on sensitivity, *i.e.* on the steady-state value of the diffusion current

3 2 3 *Influence of membrane thickness and of electrode area*

Since τ is directly proportional to the square of membrane thickness a linear plot of τ against x_1^2 should yield a straight line passing through the origin Values of τ as found in the measurements collected in Table 2 are plotted against x_1^2 in Fig 9

In practice the theory of one-dimensional diffusion in a plane sheet applies in those cases where the thickness of the sheet is very small compared to its surface area so that virtually all the

TABLE 3

mean thickness x_1 μ	d mm	x_1/d	$(\sigma \times x_1)/a$ nA/mm Hg cm	
25 0	5 0	0 0052	0 838	s.d. 0 01
	1 50	0 01 3	0 86	s.d. 0 039
	0 40	0 0618	0 949	s.d. 0,004
	0 20	0 13	1 1 4	s.d. 0.104
	0 020	1.25	8 06	s.d. 1 68
58 7	5 0	0 0117	0 853	s.d. 0,027
	1 50	0 0302	0 808	s.d. 0 025
	0 40	0 147	1 04	s.d. 0 100
	0 [illegible]0	0 204	1 548	s.d 0 192

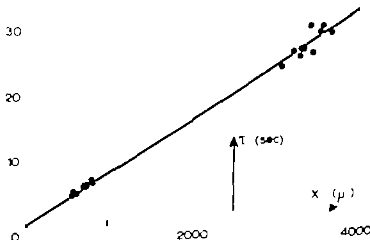


Fig 9

τ as a function of the square of membrane thickness

diffusing substance passes through the plane faces and nothing through the edges.

The results of measurements made with electrodes of 5 mm, 1.5 mm 0.4 mm, 0.. mm and 0.02 mm and with membranes of average thicknesses of ..6.9 and 58.7 μ are collected in Table 3 and

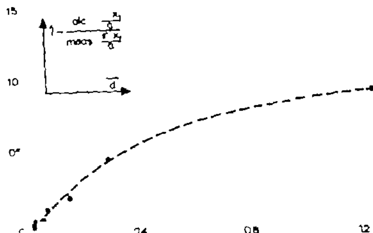


Fig. 10

Deviation of measured values of P from calculated values as a function of x_1/d.

shown in Fig. 10 where the normalized deviations of measured values of P from calculated values are plotted against x_1/d where d is electrode diameter. It will be seen that to obtain reliable results this ratio should not exceed 0.05. This limits the use of very small electrodes.

4. COMMENT

The theoretical and experimental data presented in the foregoing show that oxygen amperometry with the aid of membrane-covered electrodes according to Clark furnishes a convenient way of determining the diffusion coefficient of oxygen in hydrophobic membranes, of their permeability to oxygen and of the respective activation energies. Permeability measurements made in this way are more accurate and far less time-consuming than determinations of permeability by conventional methods.

SUMMARY

The theory of amperometric oxygen determinations with the membrane-covered Clark electrode is further developed, with particular reference to the time-course of the response. It is shown that if the contribution of the electrolyte to the response can be neglected as is the case when the membrane is pressed tightly against the electrode determination of the time in which the diffusion current reaches 61.4 percent of its steady-state value offers a convenient method to determine the oxygen diffusion coefficient in the membrane material. Since as remarked in a previous paper the membrane permeability to oxygen can easily be determined from the steady-state value of the current at known pO_2 the solubility coefficient for oxygen can also be computed, while the respective activation energies can be found from measurements at different temperatures.

Experimental data are in good agreement with the theory. Values for the permeability and the activation energy of the permeability process are in good agreement with values determined by other methods.

The solubility coefficient of oxygen in Teflon FEP is, at 25 °C, about 8 times that in water; it diminishes with increase of temperature.

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EVALUATION OF α LACTALBUMINS AND β-LACTOGLOBULINS OF COWS AND BUFFALOES BY ELECTROPHORESIS AND COLUMN CHROMATOGRAPHY

BY

R. B. MAWAL

α Lactalbumins and β-lactoglobulins are the major whey protein constituents of mature milk. ASCHAFFENBURG and DREWRY (1955, 1957a) demonstrated the presence of two genetic variants β-lactoglobulin A (β-A) and β-lactoglobulin B (β-B) in cow's milk. BELL (1962) reported the discovery of a genetically determined third variant β-lactoglobulin C (β-C) having a still slower mobility on the starch gel electropherogram than either of the two. All these polymorphic components have approximately the same molecular weight of 36 000 and each consists of two identical polypeptide chains (TOWNEND, KIDDY and TIMASHEFF, 1961) with N-terminal leucine residues and C-terminal isoleucine residues (FRAENKEL-CONRAT, 1955 and 1956; NIU and FRAENKEL-CONRAT, 1955; KALAN, GREENBERG and WALTER, 1965). Amino acid analysis has shown that the difference in electrophoretic mobilities arises from the fact that one mole each of aspartic acid and valine in β-A is substituted by glycine and alanine respectively in β-B (GORDON, BASCH and KALAN, 1961; PIEZ, DAVIE, FOLK and GLADNER, 1961) while the amino acid composition of β-C is similar to that of β-B except for a substitution of glutamic acid in β-B by histidine in β-C (KALAN et al., 1965). Buffalo β-lactoglobulin and cow β-B have approximately the same molecular weight and are practically indistinguishable in electrophoresis, ultraviolet absorption, sedimentation (SEN and SINHA, 1961b; BHATTACHARYA, ROYCHOUDHURY, SINHA and SEN, 1963) and tryptic peptide pattern (MAWAL, BARNABAS and BARNABAS, 1965), but they differ in electrophoretic behaviour in respect of boundary shapes, mobilities and charges (SEN and SINHA, 1961a).

Cow α lactalbumin is a single chain molecule of molecular weight 16 000 and its N terminal is glutamine and C-terminal leucine (WEIL and SEIBLES 1961). The polymorphism of this protein type occurs in tow breeds of African Zebus namely Nigerian White Fulani (BLUMBERG and TOMBS, 1958) and boran cattle of Kenya (ASCHAFFENBURG 1963) while it is absent in Icelandic British (BLUMBERG *et al* 1958) Danish (MOUSTGAARD MOLLER and HABSKOV-SORENSEN 1960) and North American (Holstein) cattle (PLOWMAN TOWNSEND KIDDY and TIMASHEFF 1959). BHATTACHARYA *et al* (1963) have observed *polymorphism in both* α lactalbumins and β lactoglobulins in Hariana, Sahiwal and Deshi breeds of cows in contrast to buffalo breeds where α lactalbumin and β lactoglobulin are monomorphic

SOBER and PETERSON (1954) and PETERSON and SOBER (1956) have introduced cellulose ion exchangers for the fractionation and purification of proteins. YAGUCHI, JENNINGS and TARASSUK (1959) fractionated milk *proteins* by *anion exchange* cellulose DEAE-SF (Diethyl-aminoethanol on Solkafloc cellulose lattice) and indicated that a good resolution can be obtained provided the conditions with respect to the pH nature and concentration of buffer as well as eluent are satisfied SCHOBER, HEIMBERGER and FNKELMANN (1959) reported fractionation of whey proteins into eight *distinct* fractions on a DEAE cellulose column by elution with ammonium acetate buffers. The use of DEAE cellulose columns employing gradients of sodium chloride in phosphate buffer between pH 6 0 and 6 6 for the separation of caseins into their components and whey proteins into α lactalbumin β-lactoglobulin and immune globulins is reported (YAGUCHI TARASSUK and HUNZIKER 1961 TARASSUK and YAGUCHI 1962 KIITIROU HIROSHI and KATUHIKO 1964) PIEZ *et al* (1961) have also described a procedure for the resolution of β-AB into β A and β B on DEAE cellulose columns by using linear gradients between 0 05 M sodium phosphate buffer pH 5 8 and 0 05 M sodium phosphate buffer pH 5 8 containing 0 08 M sodium chloride Recently KALAN *et al* (1965) have reported a DEAE cellulose column chromatography procedure for the separation of β-C β B and β-A with increasing sodium chloride gradient in a constant phosphate buffer (0 05 M pH 5 8) at room temperature

The present work describes typing of cows and buffaloes for

their α lactalbumin and β-lactoglobulin contents. It also describes DEAE cellulose column chromatography of α lactalbumins and β-lactoglobulins from their milks.

MATERIALS AND METHODS

The present survey on the occurrence of β-lactoglobulins and α-lactalbumins was extended to ruminants common to Western India. Two breeds of cows, namely Gir and Gavathi and two breeds of buffaloes, Jaffarabadi and Surti, were studied. Pedigree record of Gir breed cows was maintained. Milk wheys were prepared according to the method of ASCHAFFENBURG and DREWRY (1057b) with suitable modifications. The whole milk (100 ml) after treatment with anhydrous sodium sulphate and ammonium sulphate and subsequent operations was concentrated by pervaporation. The cow and buffalo breeds were typed for their constituent β-lactoglobulins and α lactalbumins by subjecting the concentrates to paper electrophoresis at room temperature at 110 V 0.05 M sodium barbitone-hydrochloric acid buffer pH 8.6 using Whatman No 3 MM paper for 16 to 17 hours. The typing procedure was applied to milk samples from 83 cows of Gir breed 50 cows of Gavathi breed and 25 each of Jaffarabadi and Surti breeds of buffaloes. Assuming that the genotypes correspond with the observed phenotypes, the gene frequencies were obtained by counting (Table 1). Milks from representative cows which contained β-A β B β-AB A B and AB were used. Since buffaloes were monomorphic with respect to α-lactalbumin and β-lactoglobulin special care in the selection of milk samples was not necessary. The α lactalbumin and β-lactoglobulins from the milks of cows and buffaloes were crystallized according to the method of ASCHAFFENBURG et al. (1057b). The β-lactoglobulins were recrystallized three times while recrystallization of α-lactalbumins was continued till they were free from serum albumin as was evident from the electropherogram.

The DEAE cellulose (Bio-Rad. Lab exchange capacity 0.65 meq/g) was regenerated by washing with 0.1 M sodium hydroxide 0.1 M hydrochloric acid and finally equilibrated with 0.05 M phosphate buffer pH 5.8. Twenty milligrams of each α lactalbumins and β-lactoglobulins were dissolved in 5 ml of water. The samples

were first dialysed against distilled water to remove adhering ammonium sulphate and then equilibrated against the starting buffer The samples were subjected to chromatography on DEAE cellulose short column (3 0 cm × 0 9 cm) at room temperature The stepwise elution of these proteins was achieved with different buffer systems (Table 2) and effluent was collected in 5 ml fractions with a flow rate of 50–60 ml per hour The concentration of the eluted proteins was determined by absorbancy measurements at 280 mμ

RESULTS AND DISCUSSION

The electrophoretic studies of both breeds of cows, Gir and Gavathi indicated polymorphism The results obtained are presented in Table 1

TABLE 1

Observed and expected frequencies of β-lactoglobulins and α-lactalbumins

(a) *β-lactoglobulin*

Breed		Total	Phenotypes			Frequency	
			A/A	A/B	B/B	A Gene	B-Gene
Gir	Obs.	83	4	3	47	0.241	0.759
	Exp		4 818	30.36	47 83		
Gavathi	Obs	20	0	11	0	0.275	0 725
	Exp		1.612	7 073	10.61		

(b) *α-lactalbumin*

Breed		Total	A/A	A/B	B/B	A Gene	B-Gene
Gir	Obs.	83	11	41	31	0 370	0.6 1
	Exp		11 05	30 08	31 06		
Gavathi	Obs	20	3	10	7	0 400	0 600
	Exp.		3.20	0 00	7 20		

The observed and expected gene frequencies of β-lactoglobulins and α lactalbumins are in good agreement with the Hardy Weinberg formula with the exception of β lactoglobulin of Gavathi breed in which case a smaller number of samples was analysed. The frequency of the A gene of α lactalbumin is in good agreement

with those observed in Hariana, Sahiwal and Deshi breeds of Indian cows, while the frequency of the A gene of β-lactoglobulin is higher than that reported in other breeds of Indian cows (BHATTACHARYA *et al.*, 1963) ASCHAFFENBURG *et al.* (1963) BLUMBERG *et al* (1958) and PLOWMAN *et al.* (1959) have reported a frequency of the A gene of β-lactoglobulin between 0.23 and 0.5 and the absence of the A gene of α lactalbumin in the breeds of European cows. Our finding that the breeds of Indian cows have a higher frequency of the A gene of α lactalbumin and a lower frequency of the A gene of β-lactoglobulin than those observed in breeds of European cows, agrees with the observation of BHATTACHARYA *et al* (1963)

No polymorphism was observed in α-lactalbumin and β-lactoglobulin in the milk of both Jaffarabadi and Surti breeds of buffaloes. Only one type each of α lactalbumin and β-lactoglobulin was present in their milk. Buffalo α lactalbumin showed a slightly higher electrophoretic mobility than that of cow α A

The stepwise elution of cow α lactalbumins and β-lactoglobulins on DEAE cellulose column was achieved with buffer systems, as shown in Table 2

The elution pattern of these proteins is represented in Fig 1

T able 2

Schedule of eluting buffers and proteins eluted.

Buffer No.	Protein eluted	Eluting Buffer
I	————	0.02 M sodium phosphate buffer pH 5.8
II	Cow α-lactalbumin B	0.03 M sodium phosphate buffer pH 5.8
III	Cow α-lactalbumin A and buffalo α-lactalbumin	0.04 M sodium phosphat buffer pH 5.8
IV	Serum albumin	0.03 M sodium phosphate buffer pH 5.8
V	Cow β-lactoglobulin B and buffalo β-lactoglobulin	0.05 M sodium phosphate buffer pH 5.8 — 0.02 M sodium chloride
VI	Cow β-lactoglobulin A	0.03 M sodium phosphate buffer pH 5.8 — 0.06 M sodium chloride

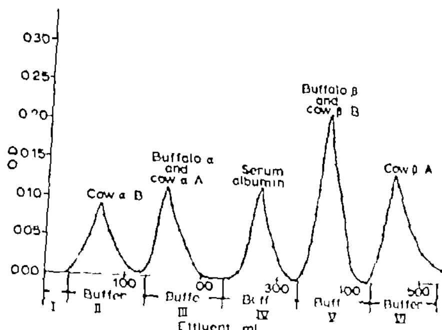


Fig 1
The effluent diagram of α lactalbumins and β lactoglobulins on DEAE cellulose column

Even though the electrophoretic mobility of buffalo α lactalbumin is slightly higher than that of cow α lactalbumin A the stepwise elution pattern of both proteins seems to be similar on the DEAE cellulose column The cow β lactoglobulin B and buffalo β lactoglobulin are similar in having the same electrophoretic mobility and elution pattern on DEAE cellulose columns

The tryptic-chymotryptic peptide pattern analyses of native cow α lactalbumin A cow α lactalbumin B and buffalo α lactalbumin have shown that these protein species have differing peptides Similarly S-sulphonated derivatives of cow β lactoglobulin B and buffalo β-lactoglobulin were found to differ in their tryptic-chymotryptic peptide pattern (unpublished)

SUMMARY

1 Gir and Gavathi breed of cows and Jaffarabadi and Surti breed of buffaloes were typed for α lactalbumin and β lactoglobulin contents in their milks

2. The frequency of the A gene of β lactoglobulin in both the breed of cows was slightly higher than that observed in Hariana, Sahiwal and Dehi breed of Indian cows

3. The frequency of the A gene of α lactalbumin in both the breeds of cows was in good agreement with those observed in other breeds of Indian cows.
4. N polymorphism was observed in lactalbumin and β-lactoglobulin in either of the breeds f buffaloes. The buffaloes had only one type each of lactalbumin and β-lactoglobulin.
5. The stepwise elution of cow lactalbumins A and B, cow β-lactoglobulins A and B, and buffalo α-lactalbumin and β-lactoglobulin on DEAE cellulose column wa achieved by molarity gradient in phosphat buffer pH 5.8 nd later by molarity gradient of sodium chloride in 0 03 M phosphate buffer pH 5.8.

ACKNOWLEDGEMENTS

The author is thankful to Principal T Barnabas for his keen interest. The author s grateful thanks are due to Dr J Barnabas, under whose supervision the present work was carried out during the tenure of a Senior U G C Fellowship His thanks are also due to the University Grants Commission for the award of Senior Research Fellowship

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NETHERLANDS SOCIETY FOR PHYSIOLOGY AND PHARMACOLOGY

PROCEEDINGS 7th FEDERATIVE MEETING OF MEDICAL-BIOLOGICAL SOCIETIES, AMSTERDAM, APRIL 14 AND 15 1966

E M Aarts *Stimulation of the glucuronic acid pathway after drug use*

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As is known administration of drugs such as pyrazolone derivatives and barbiturates to animals leads to an enhanced excretion of L-ascorbic acid The question arises what may be the effect of these drugs in this respect on man and guinea-pig that do not produce L-ascorbic acid It appeared that after administration of barbiturate and amidopyrine to man and guinea-pig an enhanced urinary excretion of D glucaric acid presents itself ((AARTS 1964).

D glucaric acid as well as L-ascorbic acid are metabolites of the glucuronic acid pathway and recently the enzyme system D-glucuronolactone dehydrogenase (1 1 1 70) was identified which brings about the conversion of *D glucuronolactone to* D-glucaric acid (MARSH 1963) Administration of D glucuronolactone to animals leads to an enhanced excretion of D glucaric acid and L-ascorbic acid The enhanced excretion of these acids after drug administration is explained by an enhanced offer of D glucuronic acid in the biophase (EVANS *et al* 1960)

When examining rats we did not observe any influence of puromycin and actinomycin D on excretion of the acids during stimulation by barbital This means that the stimulation of the glucuronic acid pathway a side effect of drugs which after therapeutic administration may occur in man is not founded on a *de novo* synthesis as shown for drug induced enhanced drug metabolism (ERNSTER and ORRENIUS 1965)

The excretion of L-xylulose is enhanced after administration of amidopyrine to pentosuriacs (ENKLEWITZ and LASKER, *1935*). This too can be explained by an enhanced offer of *D glucuronic* acid in the biophase In view of the enhanced excretion of D-glucaric acid after amidopyrine administration to man we may expect to find in non pentosuriacs a stimulation of D glucuronic acid metabolism via L-xylulose and the pentose phosphate pathway Since metabolites of the latter pathway are not *excreted* to a measurable extent we may speak of a hidden stimulation

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J. Admiraal *The influence of dextran infusions on the lipids of the blood plasma*

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In the period 1961–1963 the Hungarian investigators Lusztig, Józsa, Perenyeczky, Sajtos, Pataky and some others published an impressive series of articles concerning the influence of dextran infusions on the lipids and especially on the cholesterol content of the blood.

In the U.S.A. Flotte and Buxton (1963) reported that they could normalize the cholesterol content of the blood in several hypercholesterolaemic patients by giving 500 ml of a 6 % dextran solution daily for three days. The lowered cholesterol levels persisted for four to five weeks and could be kept low by subsequent infusions of only one bottle of 500 ml a month.

Also in The Netherlands a marked lowering of the blood lipids has been observed in patients treated with low molecular weight dextran. In one patient, given two bottles a day of 500 ml of a 10% solution of LMWD in saline, the total lipid content dropped from 1010 mg% to 515 mg% and the cholesterol content from 3 6 mg% to 11 mg%. Meanwhile the haemoglobin value dropped from 13.8 g% to 11.8 g% and the haematocrit from 4–5 to 37. Dilution of the blood thus appeared to be only partly responsible for the marked lowering of the lipids. After discontinuing the infusions the blood lipids gradually returned to pretreatment levels (after two weeks the total lipid content came to 875 mg% and the cholesterol to *00 mg%, the haemoglobin value and the haematocrit being normal) (Delprat and Oosterhuis).

In our laboratory we have started experiments to explain the above effect of dextran. Some preliminary results are given in the paper by Dr. Struer.

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E J Becker F Kreuzer and K. Rakusan *Sympathoadrenal response to simulated high altitude*

Department of Physiology University of Nymegen

In one of the studies of the Dutch Monte Rosa Expedition in 1963 (CUNNINGHAM *et al* 1965) it was found that at high altitude plasma levels of free catecholamines were significantly higher than at sea level It was also found that at high altitude about twice as much norepinephrine was excreted than at sea level. These findings were interpreted as evidence that exposure to low oxygen pressure produces an increased activity of the sympathoadrenal system However the question may be raised whether the higher concentrations were really caused by increased production and not by decreased metabolism of the active amines. Obviously determination of the major metabolite–hydroxymethoxymandelic acid–in the urine would throw light on this problem

In the present experiments high altitude conditions were simulated by a low pressure chamber The subjects collected urine at normal atmospheric pressure and again at reduced pressure and the samples were analysed for free cathecholamines and hydroxy methoxymandelic acid. The results showed that there was only a small increase of norepinephrine excretion at simulated high altitude but epinephrine and hydroxymethoxymandelic acid excretion increased twofold The highly significant increase in hydroxymethoxymandelic acid can be taken as evidence that more active amines were produced at reduced pressure since the only sources of this metabolite in normal healthy man are epinephrine and norepinephrine

In the present experiments epinephrine excretion increased much more than norepinephrine excretion while in the Monte Rosa study the opposite was seen This difference in excretion pattern may be explained by the differences of the type and duration of stress conditions experienced by the two groups of subjects.

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J A. Bernards and G P M. Horsten *On the effect of hypoxia on the electrical activity of the retina of the cat*

Department of Physiology and Department of General Physiology University of Nijmegen

Practically all information concerning the relationship between oxygen supply and electrical activity of the retina depends on experiments with ischemia of the eye (HORSTEN and WINKELMAN 195? and 1958 BORNSCHEIN 1958 BÖÖK *et al.*, 1963) In order to get more information about the role played by the oxygen lack, 10 cats anaesthetized with sodiumpentobarbital were artificially ventilated with nitrogen. In about half of these experiments the amplitude of the *b*-potential of the ERG showed an initial rise. Then in all cases the *b*-potential declined, while the *a*-potential increased. Then the *b*-potential disappeared leaving only a deep *a* potential the latter being gradually replaced by a slow negative potential. Finally this slow potential disappeared, too. From these results and also from determination of the critical fusion frequency it appears that the scotopic system is more sensitive to asphyxia than the photopic system In another 4 experiments the cats were artificially ventilated with rebreathing and CO_2-absorption. Thus the P_{O_2} gradually decreased. P_{CO_2}, P_{O_2} and arterial pressure were recorded continuously ERG was recorded separately at one minute intervals (Flash 1 6 Joule). At the moments when changes in the ERG were observed arterial blood samples were taken for measurements of P_H P_{O_2} P_{CO_2} and haematocrit. It was found in these experiments that a fall of P_{O_2} up to 1 mm Hg caused a decrease of the *b*-potential of only -5% unless the circulation remained intact When the circulation began to fail, as was shown by a sudden drop in blood pressure, the *b*-potential disappeared within one or two minutes, leaving only a deep *a* potential, which gradually decreased as described above within about half an hour after cessation of circulation.

We may conclude that in suffocation experiments the changes in the ERG are not primarily caused by a decrease in P_{O_2} of the arterial blood. but rather by a decrease in arterial pressure resulting in ischemia of the retina.

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B Bink B D R. Kluver P van Leeuwen and H. van der Sluys *Physical activity smoking and lung function*

Netherlands Institute for Preventive Medicine Leiden

This is an addendum to the epidemiologic study feeding and atherosclerosis by the Nutrition Council of the Netherlands, carried out in Zutphen by Prof Dr F S P van Buchem in 1965 for the sixth time The subjects were 717 men aged between 46 and 66

The caloric expenditure per day was estimated from a questionnaire [1]) the smoking habits were recorded according to the W H O cardiovascular questionnaire and various lung function variables were obtained by spirometry

Table 1 (p 333) shows the effects on lung function of all the other variables obtained by linear multiple regression

The multiple correlation coefficients of the lung function variables with age and height are only slightly less than those for all the variables (given in the right hand column) It therefore appears that the prediction of vital capacity etc from age and height cannot be improved by taking the remaining variables into account and remains rather unsatisfactory

1) BINK B F H. BONJER and H. VAN DER SLUYS Assessment of the energy expenditure by indirect time and motion study To be published in Ergonomica.

R A. Binkhorst and J A Vos *Metabolism and respiration during severe muscular exercise*

Department of Physiology University of Nymegen

Experiments of ÅSTRAND and SALTIN (1961) were extended by

TABLE I

The partial regression coefficient and their standard errors

		age	height	weight	inhalation	smoking	number of cig.	number of year smoking	kcal/day	multiple cor
VC	b	−0.277 ‡ 0.065	+0.550 ‡ 0.011	−0.017 0.034	+0.106 0.575	+1.301 0.681	−0.320 0.235	−0.525 0.365	+0.117 0.061	0.567
FEV	b	−0.306 ‡ 0.054	+0.347 ‡ 0.047	+0.015 0.036	+1.376 0.610	−0.074 0.723	−0.545 0.249	−0.457 0.387	+0.094 0.064	0.450
FEV in % of VC	b	−0.142 0.100	−0.273 ‡ 0.081	+0.074 0.062	+3.461 ‡ 1.047	−2.436 1.239	−0.621 0.427	−0.098 0.664	+0.003 0.110	0.199
FEV	b	−0.323 ‡ 0.053	+0.267 ‡ 0.042	+0.064 0.033	−0.463 0.551	+1.278 0.653	−0.483 0.225	−0.640 0.350	+0.048 0.058	0.490
FEV in % of VC	b	−0.203 ‡ 0.066	−0.280 ‡ 0.060	+0.231 ‡ 0.063	−1.400 0.893	+1.463 1.057	−0.603 0.365	−0.776 0.567	−0.067 0.094	0.258

= significance at 5% level; † = significance at 1% level; ‡ = significance at 0.1% level.

including load levels that could be sustained for only about 1 minute After a warming up period at a load of 150 Watt, the male subject (aerobic capacity of 4 8 l/min) performed work at loads of 300 350 400 450 500 550 and 600 Watt. Oxygen uptake was measured continuously during the work by means of the Douglas bag and Haldane method The amount of anaerobically produced energy over each working time was calculated (net efficiency 23%) Blood lactate concentrations were calculated on the basis of excess CO_2 (BOUHUYS *et al* 1966) The data were *substantially the same as those of* ÅSTRAND *et al.* (1961)

RESULTS AND INTERPRETATIONS

1 The accumulation curves for the actual transported volume of oxygen in the time revealed a transport of about 3 litres of oxygen in the first minute of exercise at a load of 300 Watt up to a definite maximum of about 4 litres of oxygen at the three highest loads 2 Working times respectively were 7 27 3 01 1 46 1.20 1 07 1 02 and 0 87 min The relation between the *logarithms* of the working times and the work loads was curve-linear confirming *the results obtained by* WILKIE *(1960)* 3 *It can* probably be concluded from the relation between percentage of the anaerobically delivered energy and the work loads, combined with the data of the high levels of the lactate concentration in the blood and the short working times that the subject at work loads of 400–600 Watt was forced to stop the work because of a high level of acidosis in the leg muscles However this hypothesis ought to be confirmed by measurements of the pH in the muscles *in vivo* 4 The relation *of tidal volume and respiratory frequency at the one* hand and *ventilation per minute at the other hand showed a distinct difference* between steady-state values and the values obtained during severe exercise Probably there is a difference in the effectiveness of the several stimuli regulating respiration at the steady-state loads and the non-steady-state severe loads.

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J Th F Boeles G J Kloosterman Marijke C. Plantema and T B Vree *Mechanical activity of the human gravid and non-gravid uterus in vitro*

Department of Physiology and Department of Obstetrics and Gynaecology University of Amsterdam

Strips were excised from the cervix and corpus of 80 non-gravid and 3 gravid (19 35 and 38 weeks resp) human uteri which had been removed for a variety of reasons. The ages of the hysterectomized patients ranged between 35 and 76 years (BOELES and KLOOSTERMAN 1965) The strips were suspended in oxygenated Tyrode s solution at 37° C and their spontaneous contractions were isometrically recorded with Sanborn inductive displacement transducers.

In contradistinction to transverse strips from the inferior part of the cervix, sagittal cervical strips and transverse strips of the upper and middle cervical regions of both pregnant and non pregnant uteri contained contractile elements (DANFORTH 1954)

Nor-adrenaline (5×10^{-6}) adrenaline (5×10^{-6}) acetylcholine (5×10^{-6}) oxytocin (3×10^{-3} I U) and pituitrin (3×10^{-3} I.U) increased the maximal contractile tension the frequency and, occasionally the tone of spontaneously active strips taken from the non-pregnant uterus and from the gravid corpus. The gravid cervix responded only to oxytocin and pituitrin.

Dihydro-ergotamine ($\times 10^{-6}$) had no effect at all on pregnant and non pregnant tissues (ROTHLIN and BERDE, 1954), whereas propranolol (10^{-6}), isoxsuprine ($\times 10^{-6}$) C C 25 ($\times 10^{-6}$) and papaverine (5 10^{-6}) decreased amplitude frequency and tone of these tissues. Papaverine appeared to be the most potent inhibitor

Isoprenaline (10^{-6}) decreased the values of these three parameters only in the gravid corpus, which effect was counteracted by propanolol Dihydro-ergotamine and regitine abolished the oxytocic effect of nor-adrenaline

From our results it appeared that there are no qualitative differences between the contractile properties of pregnant and non pregnant human myometrium.

The gravid uterus contains both α and β receptors whereas the former predominate in the non-gravid myometrium.

including load levels that could be sustained for only about 1 minute After a warming up period at a load of 150 Watt the male subject (aerobic capacity of 4 8 l/min) performed work at loads of 300 350 400 450 500 550 and 600 Watt. Oxygen uptake was measured continuously during the work by means of the Douglas bag and Haldane method The amount of anaerobically produced energy over each working time was calculated (net efficiency 23%) Blood lactate concentrations were calculated on the basis of excess CO_2 (BOUHUYS *et al* 1960) The data were substantially the same as those of ÅSTRAND *et al* (1961)

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caudal thalamic area or on other structures, since pathways from the mid-brain reticular formation to the limbic cortical areas were also destroyed by the lesion.

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Wied, D de, Int. J Neuropharm. 4; 157 (1965)

G A. Charbon *Influence of the autonomous nervous system on histamine-induced gastric secretion in dogs*

Department of Pharmacology Faculty of Medicine University of Utrecht

A quantitative study of the parasympathetic and sympathetic influences on histamine-induced gastric secretion was conducted in trained dogs with a gastric fistula. The gastric juice was collected in 3 half hour periods after administration of the drugs. When two drugs were used, these were given simultaneously

Insulin was given to stimulate via hypoglycemia, and atropine was injected to depress the parasympathetic tone while dihydroergotamine (DHE) or double-sided splanchnicotomy were employed to block the sympathetic influence on the stomach.

Insulin DHE or splanchnicotomy augmented histamine-induced gastric secretion even when maximal stimulating amounts of histamine were administered. Atropine methyl nitrate diminished the gastric secretion after stimulation with histamine or insulin. The conclusion seems justified that the gastric secretory response to stimulation with histamine varies with the tone of the parasympathetic or sympathetic innervation of the stomach.

E. N Chin A Paw P C M. Goosen and E. L. Noach *Mechanism of tyramine tachyphylaxis in the reserpinized pithed rat after loading with norepinephrine (NE)*

Department of Pharmacology University of Leiden

In pithed rats pretreated with reserpine (2 mg/kg i.p 40 and 16 h before the experiment) pressor effects of tyramine (50 µg i.v) were markedly enhanced after i.v NE infusions of 1 10 or 20 µg respectively this was in accordance with results obtained by Burn and Rand (1958) The height of the first post-infusion

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BOHUS, J TH. F., and G J KLOOSTERMAN Acta Physiol Pharmacol. Neerl 13 187 (1965)
DANFORTH D N Amer J Obstet Gynec. 68; 1261 (1954)
BOTHLIN E. and B BERDE, Helv physiol pharmacol Acta 12; 191 (1954).

B Bohus and D de Wied *Effect of α MSH on extinction of a conditioned avoidance response in posterior lobectomized rats and in rats bearing lesions in the mid-caudal thalamus*

Department of Pharmacology Medical Faculty University of Utrecht Vondellaan 6 Utrecht

The removal of the posterior and intermediate lobe of the pituitary (posterior lobectomy) in rats facilitated the rate of extinction of a conditioned avoidance response without affecting avoidance learning Pitressin purified lysine vasopressin synthetic α MSH and ACTH (A_1 peptide) administered as long-acting preparations during the period of extinction inhibits the rapid rate of extinction (of the avoidance response) in posterior lobectomized rats (DE WIED 1965)

These results suggested an important role of pituitary peptides in the CNS It was therefore deemed of interest to investigate the locus of action of these peptides

Since the literature indicates the importance of the thalamic reticular system in learning and retention of a conditioned response, lesions were placed stereotaxically in the mid caudal thalamus.

It was found that bilateral lesions in the mid-caudal thalamus destroying the parafascicular nucleus did not interfere with the acquisition of avoidance conditioning but facilitated extinction in a similar way as in posterior lobectomized rats

Since it had been shown that α MSH inhibited the rapid rate of extinction of the avoidance response in posterior lobectomized rats the effect of α MSH on extinction was studied in the lesioned rats

It appeared that α MSH did not inhibit the rapid rate of extinction of the avoidance response in rats bearing bilateral lesions in the thalamic parafascicular nuclei These results are interpreted to indicate that the action of α MSH and related peptides in avoidance conditioning may be located in the mid

caudal thalamic area or on other structures, since pathways from the mid brain reticular formation to the limbic cortical areas were also destroyed by the lesion.

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B Bohus and D de Wied *Effect of α MSH on extinction of a conditioned avoidance response in posterior lobectomized rats and in rats bearing lesions in the mid-caudal thalamus*

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The removal of the posterior and intermediate lobe of the pituitary (posterior lobectomy) in rats facilitated the rate of extinction of a conditioned avoidance response without affecting avoidance learning Pitressin purified lysine vasopressin synthetic α MSH and ACTH (A_1 peptide) administered as long-acting preparations during the period of extinction inhibits the rapid rate of extinction (of the avoidance response) in posterior lobectomized rats (DE WIED 1965)

These results suggested an important role of pituitary peptides in the CNS It was therefore deemed of interest to investigate the locus of action of these peptides

Since the literature indicates the importance of the thalamic reticular system in learning and retention of a conditioned response lesions were placed stereotaxically in the mid-caudal thalamus.

It was found that bilateral lesions in the mid-caudal thalamus destroying the *parafascicular nucleus did not interfere* with the acquisition of avoidance conditioning but facilitated extinction in a similar way as in posterior lobectomized rats.

Since it had been shown that α MSH inhibited the rapid rate of extinction of the avoidance response in posterior lobectomized rats the effect of α MSH on extinction was studied in the lesioned rats

It appeared that α MSH did not inhibit the rapid rate of extinction of the avoidance response in rats bearing bilateral lesions in the thalamic parafascicular nuclei These results are interpreted to indicate that the action of α MSH and related peptides in avoidance conditioning may be located in the mid

successively incubating Sarin (10^{-4} M) with rat plasma for 4 min at 25° lyophilizing the solution and collecting the volatile components in vacuo

The reaction of the Sarin, thus obtained, with purified bovine red cell acetylcholinesterase (AcChE, E.C.3 1 1) was studied as described by OOMS (1961) and compared with that of racemic Sarin. According to MICHEL (1955) only one enantiomer of Sarin is highly reactive towards AcChE. Our experiments demonstrated that the (−)-Sarin inhibits the enzyme faster and to a larger extent than (±)-Sarin under identical conditions, indicating that the levorotatory isomer is the better inhibitor of AcChE.

Owing to the greatly differing rate constants no appreciable amounts of AcChE inhibited with (+)-Sarin could be obtained. According to BERENDS (1964) both isomers of Sarin react with butyrylcholinesterase (BuChE, E.C.3 1 1.8) at an approximately equal rate. Therefore this enzyme, purified from horse serum has been used for our studies on the reactivation by excess of isonitrosoacetone of the (−) and (±)-Sarin inhibited enzyme (cf BERENDS, 1964) In both cases a fast initial reactivation followed by a slower reaction was observed. The results were compatible with two simultaneous and independent pseudo first order reactions. The preparation inhibited with (±)-Sarin consisted of approximately equal amounts of the fast and the slowly reacting component, while the (−)-Sarin inhibited enzyme contained about 83% of the rapidly reactivatable form. Also the ageing (the gradual loss of the susceptibility to reactivation by oximes) of (−)- and (±)-Sarin inhibited BuChE was studied the former showed the faster ageing. Our experiments indicate that (−)-Sarin is the more active isomer as far as the inhibition of AcChE and the reactivation and ageing of inhibited BuChE are concerned while it is the less susceptible form with regard to hydrolysis by sarinase.

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tyramine response was correlated with the total dose infused but the effect of the third injection was equal to that of the pre-infusion injection This complete tachyphylaxis was also present after catechol-o methyl transferase inhibition by pyrogallol and therefore could not be due to excessive leaking of the infused NE from the intracellular store to the blood otherwise re-uptake of NE and hence delayed tachyphylaxis could have been expected.

MAO inhibition by Isocarboxazide (Marplan[R]) markedly inhibited the appearance of tachyphylaxis Thus tyramine tachyphylaxis is obviously due to rapid MAO mediated breakdown of the infused NE This conclusion could be corroborated by the finding that no tachyphylaxis for tyramine occurs if in reserpinized animals an α methyl NE (Corbasil[R]) infusion is given instead of NE. Corbasil is no substrate for MAO and tyramine enhancement is still present 2 h after a corbasil infusion of 25 μg Acute pressor *effects of corbasil are 2 5 times smaller than those of NE*

Isocarboxazide did not cause immediate changes in tyramine pressor effects in reserpinized rats However a markedly increased effect was seen if the first tyramine injection was delayed for more than 40 min after MAO inhibition This enhancement was positively correlated with the time lapse This proves continuous synthesis of NE under the experimental conditions used

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P J Christen F Berends and E M Cohen *The influence of stereoisomerism of sarin on various reactions with sarinase and cholinesterase*

Medical Biological Laboratory of the National Defense Research Organization TNO Rijswijk (Z.H.)

The cholinesterase inhibitor Sarin (isopropyl methylphosphono fluoridate) is hydrolysed by an enzyme present in rat plasma the so-called sarinase (CHRISTEN and COHEN 1963) This sarinase preferentially catalyses the hydrolysis of the dextrorotatory isomer (CHRISTEN and VAN DEN MUYSENBERG 1965) An aqueous solution containing mainly the less susceptible (−)-Sarin was obtained by

M. A. Corner *Electrophysiological parameters of cerebral development in the chick embryo*

Netherlands Central Institute for Brain Research Amsterdam

Electrical activity begins during stage 42 of Hamburger and Hamilton (about 15 days in ovo) and consists of waves occurring in sequences of variable duration The distribution and the sequence of waves according to amplitude and duration indicate some essentially random underlying process. The 'direct cortical response" (DCR) begins earlier and up to stage 4_ consists of simply a surface negative wave of 5–10 milliseconds duration. In stage 43 a longer negative component appears, following the initial spike while large surface-positive waves appear in the EEG These waves occur in an essentially random sequence and cause the amplitude distribution to become bimodal. No significant changes in the electrical pattern have been observed over periods of several hours, nor is the EEG in any way responsive to sensory stimulation.

There is a strong quantitative development during stage 43 and thereafter little change until the day of hatching (stage 45) At this time a long-lasting positive wave appears in the DCR and the EEG waves become more regular approximating the rhythmic slow waves later characteristic of the sleeping state Periodic spontaneous and sensory-evoked flattening of the EEG also appear at this time although the latter is very labile and of high threshold until the chick has begun to walk about several hours after hatching Evoked potentials to light can also first be obtained during this 'pre-hatching critical period of development.

Neither cerebral spreading depression nor convulsion could be evoked in the chick embryo

A. M. Ernst *Mode of action of apomorphine and dexamphetamine on gnawing compulsion in rats*

Department of Pharmacology University of Utrecht

In rats apomorphine has been reported to induce a compulsive gnawing behaviour which has been shown to require the presence of the corpus striatum. Experiments showed that not only

H Collewijn[1]) and A van Harreveld *Intracellular recordings during spreading depression*

California Institute of Technology Pasadena Calif U.S.A

Spreading cortical depression (S.D) is a slowly (2–4 mm/min) propagating complex of phenomena in the cerebral cortex having as major aspects depression of the electrocorticogram negative shift of steady potential increase in impedance and movement of chloride and water into apical dendrites Transient depolarization of neurons has been generally regarded as the basis of the phenomenon

It was attempted to study membrane potential changes in the cortex directly by intracellular recording Special attention was given to immobilization of the cortex Only in stable penetrations of sufficient quality the effect of electrically evoked S.D was studied In 14 neurons (average membrane potential 67 mV) S.D and its recovery were successfully recorded In every instance during passage of the S D through the area of the penetrated cell a moderate to deep depolarization was seen followed by complete recovery A quick depolarization and much slower repolarization were typical. Both phases were S-shaped their total duration was around 2 min The minimal potential varied between 0% and 74% of the original one In a few cases the extra cellular field potential was recorded during a second S D after retraction of the microelectrode just outside the cell The negative shift found under these conditions indicates that the actual transmembrane potential during S D is even lower than is demonstrated by intracellular recording alone Spontaneously firing cells became inactive just prior to depolarization During the rapid depolarization a short burst often occurred The original firing pattern as well as the corticogram recovered only several minutes after depolarization

Membrane potential changes in anoxia were recorded only in a few instances A slow depolarization was seen during the first 3 minutes followed by a quick depolarization similar to that in S D This would seem to agree with earlier observations on impedance changes.

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E. D Gerlings L. N Bouman P A. Bierstoker and G Jambroes *The effect of vagal stimulation on ventricular contractility of the isolated blood-perfused rabbit heart*

Department of Physiology University of Amsterdam

In a previous communication (GERLINGS *et al.* 1965) we have shown that vagal stimulation did not affect the maximum isometric tension in the ventricles of the Langedorff heart preparation of the rabbit, perfused with Tyrode solution. This appeared only to be true if the ventricles were paced at a fixed rate

BLINKS and KOCH-WESER (1963) have suggested that changes in myocardial contractility may occur even if the "height" of the isometrically recorded contractions remains the same We have followed the authors suggestions and we have additionally determined both the d*p*/d*t* relationship and the time from the first detectable development of tension to the maximum tension of isometric contraction (Time to Peak Tension).

During maximal stimulation of the vagal nerves, slight changes in the parameters of contractility of the paced ventricles were sometimes observed. DE GEEST *et al.* (1965) however found considerable negative inotropic effects of vagal stimulation in the dog heart. We therefore assumed that the composition of our perfusing fluid might be the cause of this divergence

Since the Tyrode solution lacks macromolecular substances an increase in the extracellular space is inevitable. We considered the possibility that in our preparations the intramyocardial para sympathetic structures might be damaged by oedema.

In order to test this hypothesis we perfused the rabbit hearts with blood, according to the method originally described by HEYMANS and KOCHMANN (1904) The aorta of the isolated heart was connected with the carotid arteries of a donor rabbit. The coronary drainage of the isolated heart was led to a jugular vein of the donor The isolated heart remained in good condition during many hours and no oedema could be detected macroscopically

Even in this preparation, which did not show any sign of deterioration vagal stimulation caused again only minimal changes in the recorded parameters. It is concluded that even maximal vagal stimulation has no significant effect on ventricular contractility in the isolated heart.

administration of apomorphine but also dopa (after MAO-inhibition) and amphetamine caused this behaviour

However dexamphetamine although structurally related to the other compounds lacks OH-groups on the phenol ring This would appear to be at variance with our statement that the presence of OH-groups at the para and meta positions of the phenol ring are essential for inducing gnawing behaviour (ERNST 1965) The question can be raised whether the effects of apomorphine and dexamphetamine are dependent or independent of the presence of dopamine in the corpus striatum Accordingly the action of these drugs was studied in rats in which 1) the catecholamine-stores in the C N S were depleted by pretreatment with α methyl-dopa, resp α methyl tyrosine 2) MAO inhibition prevented metabolic degradation of the endogenous catecholamines. It appears that the gnawing effect of dexamphetamine is inhibited by α methyl-dopa pretreatment but only for 16–18 hours This indicates that the action of dexamphetamine is correlated with the recovery of dopamine and serotonine stores to normal levels suggesting that its action is dependent of the presence of (one of) these amines.

α Methyl tyrosine has been shown to block catecholamine synthesis without affecting serotonine levels. Our results obtained with α methyl tyrosine pretreatment show that in these conditions the effect of dexamphetamine was also inhibited. Accordingly it is concluded that the gnawing compulsion occurring after administration of dexamphetamine is caused by a release of endogenous dopamine

This conclusion is corroborated by the finding that pretreatment with the MAO inhibitor Iproniazid enhances the effect of dexamphetamine Since dexamphetamine itself is not destroyed by MAO this enhancement must arise consequent to protection of released endogenous dopamine

The gnawing provoking action of apomorphine however could not be modified by pretreatment with α methyl-dopa, α methyl tyrosine or Iproniazide This strongly suggests that apomorphine does not act via dopamine release but has a dopamine-like effect on the receptor structures

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which is fed with a constant 10 V signal C. Zero blood reflection is obtained by filling the cuvette with Indian ink and compensating the recorder to zero When a dilution curve is made the multiplier/divider output C/R_B can be recorded continuously

A linear relationship has been found between C/R_B and indocyanine green concentrations up to 40 mg/l. In experiments carried out in dogs, alinear and linear dye dilution curves were recorded simultaneously Semilogarithmic plottings of the descending limbs of both curves resulted in a straight line

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F ten Hoor and D I Mastebroek-Helder *Spectrophotometric properties of indocyanine green*

Department of Chemical Physiology University of Groningen

Indocyanine green (Fox green, Cardiogreen®) dissolved in plasma has a light absorption maximum at λ = 800 nm, near an isobestic point of haemoglobin and oxyhaemoglobin. This adds to its suitability for dye dilution studies. On the other hand there are certain disadvantages Firstly indocyanine green is contaminated with about 5 % NaI necessitating calibration of each dilution curve with the dye solution used. Secondly aqueous solutions are unstable the instability increasing as the dye concentration decreases (FOX and WOOD 1960)

To determine the possibility of using one calibration line for a large series of dilution curves a number of experiments were performed The results may be summarized as follows

1 Different portions of indocyanine green when dissolved in plasma, were found to have a light absorption maximum between λ = 795 and 805 nm.

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F ten Hoor and G A Mook *A linear reflection densitometer for indocyanine green*

Department of Chemical Physiology University of Groningen

The presence of a linear relationship between dye concentration in blood and recorder deflection is convenient for accurate calibration of dye dilution curves In both transmission and reflection densitometers the relationship between dye concentration and transmitted or reflected light respectively is alinear (EDWARDS *et al* 1963 ZIJLSTRA and MOOK, 1962) although it has proved possible to linearize transmission densitometers by electronic means (NILSSON 1963) Reflection densitometers have not been linearized but the resulting calibration problems were solved by using a dynamic calibration method (SPARLING *et al* 1960) It appeared however that there is a linear relationship between the reciprocal value of the blood reflection $1/R_B$ and the dye concentration This holds for the conventional reflectometers now in use as well as for a new infrared sensitive reflection densitometer

In this new densitometer light from two endoscope bulbs passes through a Baird Atomic interference filter (λ_{max} = [illegible]05 nm T_{max} = 70% band width = 40 nm) and a slit (cm long and 0.[illegible] cm wide) on a pvc tube (inner diameter = 0 15 cm) embedded in black perspex The reflected light reaches two silicium photocells (Siemens BPY 45) connected in parallel which are placed one on each side of the slit The angle between the cells and the plane of the pvc cuvette is 30 degrees

The output of the photocells is fed into a Kipp Micrograph BD 3 This recorder has an auxiliary slide wire from which an output signal may be taken By putting 10 V across this wire the resulting output signal is sufficient to drive the denominator input of a Philbrick Q 3 M I P multiplier/divider the numerator input of

The problems to be solved before the experiments could be started were

1 Construction of a stable multi-channel electro-magnetic flow meter
2 Construction of calibrated electro-magnetic flow probes which are stable for a long time
3. Chronically implantable catheters.
4. Good condition of the experimental animal and long use of the incorporated devices.

In the data processing of the flow pulses the aortic flow pulse is differentiated (dv/dt) as parameter for the force of contraction of the heart muscle and the coronary flow and stroke volume are integrated.

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W de Jong and S M. McLeod [1]), *Effect of autonomic blocking-agents on the cardiovascular effects of octapressin (phe²-lys⁸-vasopressin)*

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The effect of intravenous injections of 6 and 18 mU of octapressin on blood pressure, cardiac output, heart rate and circulation time was studied in male rats. The following blocking agents were used atropine sulfate dibenzyline propranolol (inderal), hexamethonium chloride and chlorpromazine. Saline served as the placebo Chlorpromazine (50 mg/kg s.c.) was administered 1 h before the experiment and these rats were anaesthetized with nembutal. The other four blocking-agents

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2 The optical density of a 1 mg % indocyanine green solution in plasma measured at the wavelength of maximum absorption in a layer of 1 000 cm (ε_{10}) was found to be 2 40 This value is the mean calculated from measurements performed in the course of 3 months using 18 different portions of dye The standard deviation of the individual determinations was 0 082 or 3 8% of the mean

3 To make up solutions of an exactly known dye concentration sterile dye portions had to be reweighed It proved possible to resterilize indocyanine green without change in spectrophotometric properties by means of aethylene oxide. From 11 portions of resterilized indocyanine green ε_{10} was calculated to be 2 38 with a standard deviation of 0 060 or 2 8% of the mean

4 A 1% indocyanine green solution in water proved to remain stable for about 7 hours

5 Some adsorption of the dye to glass (i e spectrophotometer cuvettes) was a complicating factor during the experiments.

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A H M Jageneau and W K A Schaper *Flow and pressure measurements in the unrestrained dog*

Janssen Pharmaceutica Research Laboratories Beerse Belgium

In a series of experiments on trained unanaesthetized dogs direct arterial blood pressure left coronary artery blood flow and aortic blood flow were simultaneously measured over periods of 4–8 h The technical procedures of surgical intervention and the implantation of the electro-magnetic flow probes were presented and the problems of repeated chronical measurements were discussed.

Flow measurements were done by the electro magnetic flow principle and the pressure with an electro manometer connected to a previously inserted intra arterial catheter (aorta descendens)

Blood flow was derived from the aorta ascendens and from the circumflex branch of the left coronary artery and in some cases from the common left coronary artery

the artificially induced micturition on bladder tissue or urinary tract but the electrical stimulus provoked pain in those animals with a neurological interruption below the spinal segment L 4.

The role of the autonomic nervous system with regard to the electrical induction of the contraction of the detrusor and the appearance of pain and the implications of our study for the clinic were discussed.

J J C. Koopmans *The nature and origin of cellulase in Helix pomatia*

Laboratory of Zoophysiology University of Amsterdam

The correlation of a secretory cycle in the hepatopancreas of Helix pomatia with the cellulolytic activity of its crop-juice by KRIJGSMAN (1925) made it likely that a cellulase of the animal itself was involved. FLORKIN and LOZET (1949) however isolated symbiotic bacteria assumed to be responsible for the cellulolytic activity of Helix crop-juice. A preliminary investigation by the author on the cellulolytic activity of the crop-juice during and after hibernation points to a cellulase secreted by the animal, while the results of PARNAS (1961) in treating Levantina hierosolyma with antibiotics are ambiguous as to the origin of its cellulase.

The foregoing makes it necessary to assume that the cellulases in Helix derive from two sources, as has already been suggested by MYERS and NORTHCOTE (1959) To verify this assumption an insight must be gained in the steps of cellulose breakdown by Helix.

To this end-among other experiments-the crop-juice of Helix was fractionated by gel filtration on a Sephadex G 100 column, a technique successfully applied to the fractionation of mould cellulases by PETTERSSON (1963) and SELBY and MAITLAND (1965)

The activity of the resulting fractions was assayed by estimating the amount of glucose produced per mg protein per hour from

A. cotton cellulose ground to a coarse suspension without much deterioration of its fibre structure (D P between 1000 and 3000) and B hydrocellulose prepared by reprecipitating cellulose from a solution in 65% sulphurous acid (D.P from *00 to 600 according to literature).

Two fractions could be distinguished one with a high activity toward substrate B with a K_D of 0 6, the other with a high activity

(10 mg/kg s.c.) were administered 30 min before the rats were anaesthetized with urethane The thermodilution method was used to measure cardiac output, and the blood pressure was monitored from the femoral artery

Pretreatment with some of the blocking-agents induced significant changes Dibenzyline lowered blood pressure to 49 ± 6 mm Hg (controls 108 ± 8 mm Hg) Heart rate of dibenzyline-treated and propranolol treated animals decreased to 368 ± 20 per min and 291 ± 19 per min respectively (controls 421 ± 10 per min)

The circulation time of propranolol treated rats increased to 5 7 ± 0 5 sec (controls 4 4 ± 0 2 sec)

The increase in blood pressure following octapressin was found to be dose-dependent in the saline-treated animals as well as in the rats pretreated with the blocking-agents Pretreatment with dibenzyline chlorpromazine atropine and propranolol potentiated the pressor response of octapressin Octapressin caused no changes in cardiac output or in circulation time The administration of 18 mU of octapressin decreased heart rate but this effect was not observed in rats pretreated with dibenzyline or propranolol

From these results it is concluded that the increase in blood pressure following the injection of octapressin is caused by an increase in vascular resistance in the systemic circulation

M C. de Jonge J A Kornelis en Jw van den Berg *Long term treatment of the experimental neurogenic bladder by means of electrical stimulation of the detrusor*

Laboratory of Medical Physics University of Groningen

With the aim to avoid the use of the urethral catheter during the treatment of patients with neurological dysfunctions of the urinary bladder we investigated in dogs with an artificial neurogenic bladder the possibility to provoke a satisfactory evacuation of the bladder by means of electrical stimulation

Irrespective of the sex of the experimental animals or the level of neurological lesion (spinal transection at D8-9 L1 2 transection of the cauda equina) we succeeded in achieving a complete or nearly complete micturition by electrostimulation during a period of at least 20 months

There was no indication of any harmful effect resulting from

cilia (the latter normally being of the antiplectic type in Paramecium) in viscous media are entirely different from those in non viscous media. An analysis of the micrographs reveals that in a viscous medium the ciliary beat, the amplitude being less and the beat being more symmetrical, resembles that in Opalina (SLEIGH, 196) while the co-ordination in that case is of the symplectic type. The areas of the ciliated surface in which the cilia show reversal resp activation in the normal direction in electric fields, however seem to be identical, the applied voltage per cm being alike in both cases.

The above experiments may indicate that the metachronism of the ciliary beat in Paramecium is not achieved by some internal transmission, as the direction of the metachronal waves alters when the viscosity of the medium is increased.

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J W Meilink and E L Noach *Effects of reserpine and chlorpromazine on epinephrine hyperglycaemia and body temperature in hypothermic and normothermic rats*

Department of Pharmacology University of Leiden

Adrenomedullectomized rats were pretreated with either reserpine (0 63 mg/kg i.p.) or chlorpromazine (16 mg/kg i.p) or placebo Effects of epinephrine (50 µg s.c.) on body temperature and blood sugar values were monitored for 3 hrs at environmental temperatures of *0 and 34 C

BODY TEMPERATURE

At *0° C the initial body temperature in drug pretreated animals was about 29 C and remained so when no epinephrine was administered. After reserpine pretreatment epinephrine caused a gradual temperature elevation to nearly normal values in the course of 3 hours. No elevation was observed after chlorpromazine. After

toward substrate A (K_D 1 1) The specific activities were 10 and 18 times those of the original crop juice respectively and were associated with about 16 and 7% of the total protein.

Treatment with dry Sephadex G 25 before fractionation did not abolish any of the activity of the latter fraction Its molecular weight therefore must lie between 5000 and 100 000 Further work on the specificity of these fractions is in progress.

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SELBY K. and C. C. MAITLAND Biochem. J 94; 5 8 (1965)

A KUYSSO *The co-ordination of the ciliary beat of Paramecium caudatum in electric fields*[1])

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It is well known that Paramecia of the Aurelia group in electric fields will be oriented along the lines of force (galvanotaxis) as the result of a typical division of the body cilia into a normally beating portion at the anodal end and a reversed beating portion at the cathodal end of the animal

Although in earlier investigations this phenomenon was stated by examining immobilized Paramecia in viscous media (LUDLOFF 1895 KAMADA 1931) it seems that under these conditions the division into a reversed beating group of cilia and a non reversed beating group does not essentially differ from that in freely swimming animals during galvanotaxis since PÁRDUCZ (1963) applied his instantaneous fixation technique studying Paramecia showing galvanotaxis.

In the present experiments however in which the ciliary beat of Paramecium caudatum is studied in viscous as well as in non viscous media by means of a stroboscope and of flash micrographs, it appears that the ciliary beat and co-ordination of the body

1) Supported by the Netherland Organisation for the Advancement of Pure Research (Z.W O)

composition of the medium on the force and direction of the ciliary stroke

In the present experiments the responsiveness to an electric field and the swimming-rate at different potential gradients were studied in P. aurelia which, by a stay overnight, were adapted to solutions of KCl and $CaCl_2$ in distilled water. Electrotaxis was normal in all solutions, i.e. the animals turned to and moved in the direction of the cathode. It was found that upon the reversal of the direction of the field, the time the animals require to turn is inversely proportional to the potential gradient. As a measure for responsiveness we adopted the potential difference corresponding to twice the minimum reaction time. KCl in the range of 0.1–3.0 meq/l increased the responsiveness; higher concentrations were toxic under the conditions used. $CaCl_2$ in the range of 2–3 meq/l had the opposite effect. As to the effects on the swimming rate, on the contrary, KCl had a depressive influence while $CaCl_2$ increased the rate of propulsion.

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J. Noordhoek *The relation between dose and sleeping-time after intravenous hexobarbital administration to mice*

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The relation between dose and duration of narcosis after i.v. administration of hexobarbital was studied in mice.

The relation between the logarithm of the dose and the sleeping time was found to be linear if the doses ranged from 40–80 mg/kg.

We have tried to elucidate the factors determining the slope of this dose-response line.

In general the sleeping-time after intravenous injection of hexobarbital is determined by

1. The sensitivity of the C.N.S. to hexobarbital
2. The elimination rate of the drug from the C.N.S.

placebo pretreatment, the initial body temperature was 3°° and remained unaffected by epinephrine

At *34° C* the initial body temperature in drug and placebo pretreated animals was normal (37–38) Epinephrine caused a temperature elevation of about 1 5 C, the maximum being obtained after 1 hour

BLOOD SUGAR

Reserpine pretreatment did not markedly affect initial blood sugar values at either environmental temperature. After chlorpromazine the initial values were very *slightly increased* at °0 C.

At *20° C* reserpine pretreatment caused a marked flattening of the epinephrine blood sugar curve Values after 3 h were within the normal range probably in correlation with normalized body temperature After chlorpromazine pretreatment, the epinephrine *blood* sugar curve was much higher than in controls *After* 3 h no return to normal values was apparent

At *34° C blood sugar curves in drug pretreated animals* were shifted towards normal

E D Nijenhuis B J G Flik and H J Boosewinkel

Long-term effects of Ca^{++} and K^{+} ions on the responsiveness and swimming rate of Paramaecium aurelia in an electric field

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Electrotaxis in Paramaecia exposed to an electric field is explained from a local reversal of ciliary movement due to hypopolarization of the cell membrane *in* the *area* facing the electrode (JAHN 1961) Ciliary reversal may also occur upon the transfer of Paramaecia to media with an altered ionic composition (OLIPHANT 1938) At least as far as K^{+} ions are involved this phenomenon too is attributed to changes in the potential across the membrane (YAMAGUCHI 1960 NAITOH 1958) Ciliary reversal in response to an altered medium however is a transient effect. Though in view of current knowledge of the structure of biological membranes (BOOIJ and BUNGENBERG DE JONG 1956) the medium should exert a more *persistent influence on the functions* of the cell membrane, little is known of the long term effects of the ionic

B. Oeseburg and J. Stuttorheim *Ascorbate dilution curves obtained using a single catheter for injection and measurement*

Departments of Chemical Physiology and Paediatric Cardiology University of Groningen

Reliable ascorbate dilution curves can be obtained using an intravascular platinum electrode catheter. Ascorbate is injected into the central part of the circulatory system and the indicator concentration course measured amperometrically with the intravascular electrode. No withdrawal system as used in dye dilution techniques is necessary, thus allowing for better dynamic response characteristics. Also a smaller artery than that necessary for withdrawal of blood for dye dilution curves may be used for the introduction of the platinum electrode. A linear relationship has been found to exist between the concentration of ascorbate and the depolarization current measured. Ascorbate dilution curves are thus quite suitable for evaluation of shunt blood flow using Mook's equations (MOOK and ZIJLSTRA, 1961).

To evaluate left to right shunts without puncture of an artery a commercially available platinum electrode catheter was modified. The opening at the tip of the catheter was closed with lacquer and 4 new injection openings were drilled in the catheter wall about 20 cm from the tip. Special care must be taken not to damage the connecting lead embedded spirally in the catheter wall. To this end an apparatus was constructed with which the position of the wire may be detected. The catheter is introduced into the right heart via a peripheral vein, the injection openings then being situated in the vena cava. The right ventricle acts as a mixing chamber between the injection openings and the measuring electrode.

Using this catheter, shunt blood flow was determined in 10 children and compared with the results calculated from simultaneously obtained oximetric data. This yielded a mean difference between the two methods of +2% shunt flow with a standard deviation of 5% shunt flow.

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MOOK, G. A. and W. G. ZIJLSTRA, Acta Med. Scand. 170; 703 (1961).

From the 10th minute after injection of hexobarbital the logarithm of its brain concentration (c) was observed to decline linearly with time (t) This points to a one-compartment elimination system in which the metabolism by liver enzymes plays the principal part

$$\text{So} \quad \log c = -kt + \text{constant}$$
$$\log c_0 - \log c_{t_s} = kt_s$$

if c_0 = the brain concentration extrapolated to $t = 0$

c_{t_s} = the brain concentration at awakening

t_s = sleeping time

k = elimination constant

After substitution of $c_0 = d/v$ (d = dose v = specific volume of distribution) the equation becomes

$$kt = \log d + \log v/c_{t_s}$$

If v c_{t_s} and k would be independent of the dose this equation would give a linear relation between t_s and log dose

A decrease of k and a slight increase of c_{t_s} with increasing dose however was observed in further research whereas v remained constant

Nevertheless calculations showed the second term in the equation to remain approximately constant

In the first term there must be a special relation between log dose and k which prevents the log dose sleeping time lines from becoming curved Therefore the slope of these lines may be concluded to be determined mainly by the elimination rate of the drug and only to a minor degree by sensitivity of the C.N.S.

Differences between our results and those of WINNE (1964) who found a curved log dose sleeping time line may be explained by the higher doses used and the lower environmental temperature during his experiments These resulted in a much sharper fall of the rectal temperatures of his mice during narcosis compared to ours

Preliminary investigations with log dose sleeping time lines confirmed their usefulness in elucidating the mechanism by which certain drugs lengthen or shorten hexobarbital sleeping times.

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WINNE D., Naunyn-Schmiedebergs Arch. exp Path u. Pharmak., 247: 278–294 (1964)

Three 360 ml glass tonometers containing 1 ml human plasma each, were placed in a water bath, the temperature of which was kept constant within 0 1 C. Saturated O_2/CO_2 mixtures were led for 75 min through the tonometers swinging over an arc of 1°0 degrees. During equilibration the gas mixtures were analysed with a Haldane apparatus the buret of which was calibrated by weighing. Two 4 ml plasma samples were then taken from each tonometer and pH and total carbon dioxide content $c^{P}_{CO_2}$ (tot) determined. The pH was measured using a Radiometer E 5021 microchain

TABLE 1
Apparent first dissociation constant of carbonic acid in plasma at different temperatures and pH.

	pK' (exp)	pK' (calc))	pH	n
37.5° C	6.081	6.084	7.609	21
	6.093	6.093	7 436	49
	6.108	6.106	7 183	52
35° C	6.08	6.085	7.599	10
	6.106	6.103	457	12
	6.110	6.118	7 162	12
32.5 C	6.102	6.104	7.632	11
	6.113	6.114	7 457	11
	6.119	6.128	7 191	12
30° C	6.118	6.114	7.667	18
	6 120	6.127	7.421	14
	6.145	6.141	7 151	43
25 C	6.123	6 134	7.623	21
	6 142	6.146	7 423	17
	6.153	6 160	7 143	26
20 C	6 162	6.163	7.616	18
	6 170	6 175	7.355	32
	6.191	6 190	7 120	37
15 C	6 183	6 186	7.561	20
	6.200	6.193	7.378	21
	6.211	6.212	7.057	49

) Calculated using eq. (2).

W J Riotveld W E M Tordoir and J R B Hagenouw
Attentiveness and cortical evoked responses

Psychophysiology Division Department of Physiology University of Leiden

Most authors describe an increase in amplitude of the cortical evoked potentials to lightflashes as well as to clicks when attention is focussed upon the stimuli some authors however found a decrease

Experiments have been carried out to resolve this discrepancy It was found that at low frequency stimulation the cortical responses are contaminated by an artifact due to eyeball movements and contraction of the scalp muscles In the attentive state these artifacts decrease in amplitude

Moreover it was found further that the responses (visual as well as auditory) decreased during performance of a difficult task with an easier one however there was an increase in amplitude Correlations were found between the behaviour of the evoked responses and the simultaneously recorded background EEG the decrease of the evoked responses while performing a difficult task was accompanied by a desynchronization whenever an increase occurred more alpha-spindles could be seen

P Rispens C W Dellebarre D Eleveld T M E. Fongers W Helder J A Zijlma and W G Zijlstra *Determination of the apparent first dissociation constant of carbonic acid at different temperatures*

Department of Chemical Physiology University of Groningen

There is still some disagreement on the value of the apparent first dissociation constant of carbonic acid in plasma at different temperatures Since this value is of great importance for the evaluation of the acid base status using the Henderson Hasselbalch equation experiments were performed at seven different temperatures to redetermine the value of this constant As a pH dependency of pK_1 due to pH dependent errors in some of the determinations underlying the calculation has been reported the symbol pK'_1 is used instead of pK_1

are being utilized as a functional measure of the effect of X ray exposure on the visual cortex. Twenty-one animals, 24 months of age were exposed to a single dose of 3500 rads of X rays to the right visual cortex of the occipital lobe The X-ray beam was limited to the cerebral cortex, its coverings and the immediately subjacent white matter to a depth of 1 cm. Previous to irradiation and at 24 hours 72 hours and weekly thereafter until sacrifice serial EEG and clinical examinations were performed on each animal. At 72 hours, 1 4 12 28 and 44 weeks three animals were sacrificed for microscopic and electron microscopic evaluation of changes in the irradiated and non-irradiated sides. EEG evaluation involved both visual analysis and electronic frequency analysis of the evoked response elicited by photic stimulation of the retina. Animals were subjected to a program of photic stimulation at predetermined frequencies. The resulting evoked potentials are visually examined and stored on magnetic tape for analysis by a Grey Walter type low frequency analyser Within 24 to 72 hours most animals showed a slight to moderate depression in amplitude of the photo-evoked response on the irradiated side accompanied by a lesser depression on the non irradiated side. Beyond 15 weeks this trend continued with somewhat increased variations noted between individual animals. At 4 weeks it became more difficult to elicit a clean evoked response, and, in some animals, by 25 weeks it was essentially impossible to drive the visual cortex. Microscopic examination and precise measurement of several dendritic plexus parameters indicate a significant reduction in the dendritic plexus on the irradiated side as early as 4 weeks post-irradiation, becoming more severe with time Electron microscopic findings also indicate serious structural alterations on the irradiated side which may be correlated with the observed functional alterations.

J H Schuurmans Stekhoven and F Kreuzer *Spontaneous atelectasis in the anaesthetized dog with artificial ventilation*

Department of Physiology University of Nymegen

In anaesthetized man and dog with artificial ventilation an increase of the alveolar-arterial O_2 pressure difference ($AaDO_2$) in the course of several hours has been found by various authors.

electrode with Radiometer type 4 or type 27 pH meters calibrated by means of a NBS phosphate buffer Measurement of $c^P_{CO_2}$ (tot) was performed with a photometric method (BRUNSTING 196?) calibrated with standard bicarbonate solutions. Reproducibility of the Haldane gas analysis was 0 04 vol % CO_2 of the pH measurement 0 006 and of the $c^P_{CO_2}$ (tot) determination 0 3 mEq/l pK'_1 was calculated using the equation

$$pH = pK'_1 + \log\left[\frac{c^P_{CO_2}(tot)}{\delta\, P_{CO_2}} - 1\right] \qquad (1)$$

The value reported by AUSTIN and co-workers (1963) was taken for S

In all 518 duplicate pK'_1 values were obtained with a reproducibility of 0 008 Results were divided into three pH groups at the seven temperatures used 16 20 26 30 32 5 and 37 5 C, and are presented in Table 1

The relationship between pK'_1 pH and temperature was as a first approximation taken to be of the form

$$pK'_1 = A + B/T + C\ pH \qquad ( )$$

The constants A B and C were computed with the multiple regression method to be 5 121 414 61 and −0 0530 respectively The pK'_1 values calculated using the above values for the constants are also given in Table 1 The agreement between the experimental and calculated values is quite sufficient for clinical purposes.

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J P Schadé W F Cavonoss L Roizin and A L Carsten

Modification of the photo-evoked response in the monkey by X irradiation

Netherlands Central Institute for Brain Research Amsterdam The Netherlands Dept of Neurology Columbia University New York N Y U.S.A

Changes in the photo-evoked response in the Macaca mulatta

receptor interaction is concerned. It may be possible to differentiate between more or less essential moieties in this respect, which implies a differentiation between more or less critical moieties as far as the toleration of structural changes is concerned (ARIËNS, 1966). The consequence is that the ratio for the activities of two optical isomers will depend on the location of the center of asymmetry in the drug molecule or in other words, also in highly active drugs low activity ratios may be found for the optical isomers if the center of asymmetry is located in a non-essential moiety. An experimental confirmation of this restriction to the generalization given above was obtained in the study of various series of amino esters with centres of asymmetry in the amino moiety or/and in the acidic moiety. For the anticholinergic compounds the acidic moiety was found to behave as an essential moiety, the amino moiety as a non-essential moiety (ELLENBROEK, 1964 and ELLENBROEK *et al.* 1965). Strictly taken a change in sterical structure is comparable to a structural change in general. Relations as reported for the ratios in the activities for stereoisomers could be confirmed for series of cholinergic and anticholinergic drugs by comparing compounds with an NMe_3-moiety with their analogues with an NMe_3-moiety, with an NMe_2Et-moiety, with an $NMeEt_2$-moiety or with an NEt_3-moiety (BARLOW *et al.* 1963, VAN ROSSUM 1959 and 1962, SIMONIS *et al.* 1963). On basis of these experiments it may be concluded that in the cholinergic compounds the amino moiety is an essential moiety, while in the anticholinergic compounds it is a non-essential moiety. Another restriction to the general rule may be expected if couples of stereoisomers are tested on different receptorsystems. This is so, since the degree in which particular moieties of the drug are essential for the interaction with the receptor may differ for the different receptors. The results obtained in a study on the activity of a series of optically active organic phosphates on acetylcholine esterase and pseudo-acetylcholine esterase (butyrylcholine esterase) by OOMS and BOTER (1965) may serve as a confirmation of the restriction just outlined. Although the sequence of the various compounds as far as their activities were concerned was the same for both esterases, the activity ratios on the butyrylcholine esterase as a whole were found to be lower than those on the acetylcholine esterase. If series of couples of compounds are compared for their activity on more remote receptor

This increase has been attributed to venous admixture due to progressive atelectasis resulting in a fall of the arterial O_2 pressure (P_{aO_2}) particularly in hyperoxia. The P_{aO_2} could be brought back to the original level by reversal of the atelectasis with increase of the intratracheal pressure

Supine dogs anaesthetized with pentobarbital and curarized, were artificially ventilated with pure O_2 the pump frequency was 12 per minute and the tidal volume was adjusted so as to obtain an alveolar CO_2 pressure (P_{ACO_2}) of about 35 mm Hg P_{aO_2} remained unchanged during several hours with an $AaDO_2$ of about 60 mm Hg and a venous admixture of some 3% of cardiac output There was no change in these parameters when increasing the pump frequency to 20 per minute and decreasing the tidal volume which was accompanied by a strong increase of P_{ACO_2}

Inspection at autopsy showed only a few local patches at the dorsal side of the lungs microscopic examination of the lungs did not reveal any appreciable atelectasis.

No ready explanation for the discrepancy between these findings and those in the literature can be offered In comparing the occurrence of atelectasis and its effects on P_{aO_2} the possibility of local circulatory adjustment to atelectatic regions needs further examination

A. M. Simonis and E J Ariëns *Structure and action some general principles*

Pharmacological Institute University of Nymegen

Pfeiffer (1956) reported for a variety of pharmacologically active compounds with a center of asymmetry that the ratios for the activities of the optical isomers increase with the increase in the activities that is the doses needed of these drugs. This correlation makes sense since generally one may expect that for highly active compounds which will have a high degree of complementarity with their receptors a change in sterical structure will be of more influence on the activity than in the case of less or slightly active compounds for which a poor fit or a lesser degree of complementarity with their receptor may be assumed A more detailed consideration however shows that the various parts of the drug molecule are not necessarily of equally essential importance as far as drug

receptor interaction is concerned. It may be possible to differentiate between more or less essential moieties in this respect, which implies a differentiation between more or less critical moieties as far as the toleration of structural changes is concerned (ARIËNS 1966). The consequence is that the ratio for the activities of two optical isomers will depend on the location of the center of asymmetry in the drug molecule or in other words, also in highly active drugs low activity ratios may be found for the optical isomers if the center of asymmetry is located in a non-essential moiety. An experimental confirmation of this restriction to the generalization given above was obtained in the study of various series of amino esters with centres of asymmetry in the amino moiety or/and in the acidic moiety. For the anticholinergic compounds the acidic moiety was found to behave as an essential moiety, the amino moiety as a non-essential moiety (ELLENBROEK, 1964 and ELLENBROEK *et al.* 1965). Strictly taken a change in sterical structure is comparable to a structural change in general. Relations as reported for the ratios in the activities for stereoisomers could be confirmed for series of cholinergic and anticholinergic drugs by comparing compounds with an NMe_3-moiety with their analogues with an NMe_2-moiety, with an NMe_2Et moiety, with an $NMeEt_2$-moiety or with an NEt_3-moiety (BARLOW *et al.* 1963, VAN ROSSUM 1960 and 1962, SIMONART *et al.* 1963). On basis of these experiments it may be concluded that in the cholinergic compounds the amino moiety is an essential moiety, while in the anticholinergic compounds it is a non-essential moiety. Another restriction to the general rule may be expected if couples of stereoisomers are tested on different receptorsystems. This is so, since the degree in which particular moieties of the drug are essential for the interaction with the receptor may differ for the different receptors. The results obtained in a study on the activity of a series of optically active organic phosphates on acetylcholine esterase and pseudo-acetylcholine esterase (butyrylcholine esterase) by OOMS and BOTER (1965) may serve as a confirmation of the restriction just outlined. Although the sequence of the various compounds as far as their activities were concerned was the same for both esterases, the activity ratios on the butyrylcholine esterase as a whole were found to be lower than those on the acetylcholine esterase. If series of couples of compounds are compared for their activity on more remote receptor

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J W Snellen *Negative work during walking and cycling on a motor-driven treadmill*

Department of Physiology University of Nymegen

The oxygen expenditure of walking downhill and cycling uphill and downhill at different pedalling speeds and transmission ratios has been investigated in order to find the largest negative load sustainable during a period of an hour with the smallest increase in metabolic rate

The smallest increase in metabolic rate was found in cycling with high pedalling speed (80 r p.m.) The sustainable work load is largely dependent on the subject but negative loads of * kcal/min with metabolic rates of 3 kcal/min are practicable

The findings are in accordance with the literature

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W F H Stroër *On the interaction of dextranmolecules with plasma proteins and cholesterol*

Poviet Producten N V Amsterdam

It is possible to lower a high lipid-contraction of the blood by administering a dextran solution to the organism however the mechanism of this action is not known.

Since RICKETTS (1952) showed that dextran forms complexes with fibrinogen, several investigators verified the same for nearly all plasma protein fractions.

It can easily be confirmed by the sedimentation, noticed after mixing a 2 % solution of any of these fractions .5 1 with dextran. But the question in how far impurities of the isolated protein fractions are responsible for the phenomenon, remains open.

For a more exact approach an analysis of the sediments, obtained by mixing plasma with dextran was tried out by means of EE and IE but, for some unidentified reason, without success For this reason an antiserum against this sediment (obtained by mixing bovine plasma with dextran) was developed in the rabbit It could be shown that the sediment chiefly consists of fibrinogen and also of haptoglobulin and γ-globulin.

systems such as for instance the muscarinic and nicotinic receptors, also the sequence of compounds in the series as far as their activity is concerned is found to be changed.

In conclusion no correlation between the ratios for the activities of couples of related compounds such as stereoisomers and the activity of the most active compound of each couple is expected, if a variety of drugs acting on a variety of biological systems is involved. In other words the clear cut correlation observed by Pfeiffer might well be incidental

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W A Smit G Becht and A. M Th Boenakkers *Correlations between structure and function in insect muscles*

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Zoological Institute Catholic University of Nymegen

Extensors trochanteres of the mesothoracic leg of the cockroach Periplaneta americana were studied

Correlations were found between (a) the structure the colour and the number of sarcosomes, (b) some enzymes of the energy producing pathway and (c) the staying power

It was concluded that mechanical functions of muscle are graded by internal properties of the fibres as well as by neuromuscular control

J. C. de Valois *Some aspects of oxygenation at the capillary level*

Netherlands Central Institute for Brain Research Amsterdam

A critical analysis was made of the process of oxygen transport in the CNS. This process, oxygen diffusion, appears to be dependent on a number of factors which are not readily accessible either by theoretical or by experimental approach. Some of these factors, such as the number of capillaries supplying one single neuron and certain characteristics of capillary networks, were elucidated. The value obtained from the literature for the intracellular oxygen tension below which cellular oxygen consumption decreases varies from about 10 mm Hg to 0.6 mm Hg. For the determination of the oxygen diffusion coefficient, dead tissues at lower temperatures than in the *in vivo* condition have been generally used and the enormous changes occurring in dead or dying tissue have been insufficiently taken into account. Especially in regard to ionic movements and the concomitant changes in intra- and extra-cellular space, it will be evident that any comparison between *in vitro* and *in vivo* conditions is rather precarious. The "cylinder" model for oxygen diffusion, introduced by Krogh and elaborated by Thews *et al.*, is far too simple and can no longer be regarded valid.

P. E. Voorhoeve, P. Andersen and B. Holmqvist *Excitatory synapses on hippocampal apical dendrites activated by entorhinal stimulation*

Accepted for publication by Acta Physiologica Scandinavica.

J. Vos and H. J. van der Helm *Proteins of the developing brain*

Netherlands Central Institute for Brain Research and Neurology Department, Wilhelmina Gasthuis, Amsterdam

From the data published by LAJTHA (1964) it can be concluded that cerebral proteins of young animals have a fast overall turnover rate compared to those of older animals. In order to determine whether this could be caused by differences in protein composition, the pallium of rabbits of 0, 5, 10, 15, 20 and 30 days of age and the hemisphere of chicken embryos of 6, 12, 14, 16, 18 and 20 days and of hatched chicks of 3, 7 and 10 days were investigated by

This was confirmed by an IE of the supernating fluid after removal of the sediment Here the just-mentioned fractions were lacking or diminished. Less convincing were the results of a planimetric analysis of quantitative IE s obtained by a technique developed by AFONSO (1964) It was found that after mixing with NND-dextran and with dextran BP only the α_1-globulins remained unchanged but that the concentration of all the other protein fractions especially of β_1 (fibrinogen?) and of γ-globulin were lowered More experiments have to be performed to confirm these results

These observations cannot explain the lowering of the lipid concentration by dextran

Now it is well known that cholesterol and phospholipids are especially bound to the α_1 and β_1 lipoproteins. We could show that in bovine plasma the first and in rabbit plasma the second are the most important. In man the lipid-containing fractions were confined to the pre beta or alpha 2 lipoproteins (LEVY c.s.)

Now when dextran was mixed with plasma of man cattle or rabbit and the sediment was removed by centrifuging in none of these lipoproteins a decrease could be observed by FF or by IE. The plasma was used untreated as obtained after removal of the erythrocytes or after raising the cholesterol content by mixing it with this lipid in combination with Tween 80

Since dextran did not react with esterified cholesterol another possibility had to be verified *i e* the reaction between dextran and free cholesterol

To investigate this cholesterol was dispersed in distilled water with the aid of Tween 80 The fluid was centrifuged for 30 min to remove the excess of cholesterol and then distributed among several jackets. To each of them a solution of dextran of different Mw and concentration and to the control 0 9% NaCl 1 4 was added With the exception of the control fluid a flocculous sediment was formed in all mixtures After centrifuging for another 30 min the control fluid remained opaque but all the others were more or less transparent with a sediment on the bottom

This experiment shows that dextran combines with free cholesterol and by this way lowers the lipid content of the plasma.

Further investigations are being planned to substantiate this conclusion and to find out what happens with these sediments.

CO_2-content. The resulting galvanometer deflections of the diapherometer were recorded using a Kipp Micrograph BD with automatic channel selector

Because CO_2 is absorbed before the gas mixture reaches the O_2 detector in the diapherometer the sensitivity of this detector can be easily calculated from recorder deflection and change in O_2 concentration. The recorder deflection caused by the CO_2 detector however is determined by the detector sensitivity for both O_2 and CO_2. By measuring expiratory air containing different O_2 and CO_2 concentrations, pairs of equations are obtained from which the separate sensitivities may be calculated. In one series of experiments all or part of the CO_2 of the expiratory air was absorbed to increase the differences between the gas mixtures led into the diapherometer

Using the thus determined sensitivity values oxygen uptake $\dot{V}_{O_2}$ and carbon dioxide production $\dot{V}_{CO_2}$ were determined with the diapherometer and calculated from simultaneously performed Haldane analyses. The reproducibility of $\dot{V}_{O_2}$ was 1 17% for the diapherometer and .77% for the Haldane analyses. These values are 1.28% and 1 0*% respectively for $\dot{V}_{CO_2}$. The mean difference between the diapherometer and the Haldane values was only slight. These results indicate that the diapherometer is sufficiently accurate to be used in the determination of cardiac output according to the Fick principle

D de Wied *Effect of autonomic blocking-agents on the salt arousal of drinking in the rat*

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The placement of minute amounts of cholinergic agents in the hypothalamus of water-satiated rats causes vigorous drinking, indicating that cholinergic stimulation participates in the regulation of water intake (GROSSMAN 196_). Accordingly it seemed of interest to investigate whether other stimuli for water intake would be mediated by similar cholinergic mechanisms in the CNS The salt arousal of drinking' (Adolph) as induced by the subcutaneous injection of a hyperosmotic NaCl solution (WAYNER *et al.* 1964) produces vigorous drinking in rats, which remains constant for

means of polyacrylamide gel electrophoresis. In the developing pallium of the rabbit 21 fractions were found and ?0 fractions in the hemisphere of the chick After addition of Triton x 100 to the gels and extracts 2 extra fractions were found to enter the gel. During development only 2 fractions of the rabbit pallium showed an increase in concentration while no changes were observed in the remaining fractions In the developing hemisphere of the chicken 5 fractions showed an increasing concentration while 2 fractions decreased At 14 days of incubation a fraction appeared while at 20 days of incubation a fraction disappeared It seems that only minor changes occur in the soluble protein composition of the developing rabbit pallium In the brain of the pre-hatched chick more profound changes take place in its protein composition

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E van de Wall P Rispens F ten Hoor and W G Zijlstra

Calibration of the diapherometer with expiratory air

Department of Chemical Physiology University of Groningen

As the Noyons diapherometer (Kipp Delft) widely in use for metabolic rate measurements does not impose any burden on the patient the method was supposed suitable for the determination of oxygen uptake and carbon dioxide production for cardiac output determinations according to the Fick principle in cases of cardio-pulmonary disorders A study of the accuracy of the method was therefore undertaken

Calibration of the diapherometer is usually performed by leading pure O_2 and CO_2 through the hood However because gas cylinders proved to contain traces of Ar and Kr and to simulate the conditions under which the measurements take place in clinical practice it was decided to calibrate the diapherometer with expiratory air To this end expiratory air from volunteers was collected in a 1300 l airtight pvc bag This pvc bag is connected via a calibrated rotameter and an airtight centrifugal pump to the hood of the diapherometer Samples could also be led simultaneously to a Haldane gas analysis apparatus for the determination of O_2 and

To determine whether this effect of DFP is due to a direct effect on the heart, experiments were carried out with the isolated rat heart according to Langendorff. The dependency on the dose of DFP and the effects of atropine and P_2S were investigated. It was found that in the isolated heart DFP can induce effects similar to those found in vivo. The degree of reduction of ventricular contraction force and of millivoltage of ECG (peak to peak values) in the course of the experiments depends on the dose of DFP. This loss of function cannot be influenced by either atropine or oximes. Using several dose levels it was found that perfusion of the heart with 3 μg DFP/ml tyrode during 5 min caused effects which already differed significantly from untreated control hearts. This threshold dose was comparable to the threshold dose *in vivo*, since it was observed that after injection of $4 \times LD_{50}$ DFP s.c. in intact animals blood levels of "free" DFP between 3.5–5.5 μg/ml blood were found to occur during some 10 min. The dose of $4 \times LD_{50}$ may be considered as a threshold dose *in vivo* since with this dose 4 out of 6 animals died from heart failure; after higher doses all rats die.

The phenomenon of cardiac death may also be observed after organo-phosphates other than DFP, such as sarin.

Cardiac death may be important in so far as it determines the upper limit of successful therapy with oximes and atropine; after DFP this upper limit is reached with comparatively low doses.

several hours. Therefore the effect of a variety of centrally acting substances mainly related to autonomic blocking agents was studied on the salt arousal of drinking in order to gain an insight into the pathways that participate in this process of water consumption.

Male white rats weighing 180–220 g were injected subcutaneously with either 1 2 ml of a 15% NaCl or of a 0 9% NaCl solution *containing 2 4% procaine HCl to avoid pain* The animals were placed into metabolic cages and water intake was measured every hour Since maximal drinking was obtained within 2 hours after loading with NaCl in subsequent experiments the intake was studied for 2 hours Water intake of rats treated with 0 9% NaCl amounted to 1 7 ± 0 9 ml that of animals treated with 15% NaCl to 9 4 ± 0 9 ml per 2 hours Autonomic blocking agents and related substances were administered intraperitoneally at the same time of the injection with the NaCl solutions.

Scopolamine methamphetamine amphetamine chlorpromazine atropine mecamylamine hexamethonium nethalide inderal cocaine *dimpal artane and neobenodine marshalled in the order* of potency blocked or reduced the salt arousal of drinking

Methylatropine nitrate dibenzyline cyproheptadine deseril and pitressin tannate in oil failed to affect the increased water intake of thirsty rats.

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O L. Wolthuis and F Meeter *The effects of DFP on the rat heart*

Medical Biological Laboratory of the National Defense Research Organization TNO Rijswijk (Z H)

Atropinized anaesthetized rats treated with 4 8 or 16 × LD_{50} DFP subcutaneously die from heart failure notwithstanding continuous artificial respiration or treatment with oximes (P_2S or *LüH₆*) The time of death after injection of DFP depends on the dose of the organophosphorous compound Even complete recovery of neuromuscular transmission and return of spontaneous respiration after oxime administration does not prevent from death of cardiac failure

Eugene J Becker *Catecholamine metabolism*

Department of Physiology University of Nijmegen

It is well known that all physiological stimuli result in changes of the level of activity of the sympathoadrenal system. Measurement of the concentrations of the compounds involved in these changes can give information about the events taking place when the organism is exposed to such stimuli.

The biosynthesis and metabolism of adrenaline (A) and noradrenaline (NA) are fairly well understood but there is no complete agreement with regard to the production plasma levels and excretion of the active amines and their metabolites. According to the best available sources, the healthy adult human during a moderately active 24 h day produces or releases about 1–2 milligrams of A and about 4–8 milligrams of NA. Practically all the A is secreted by the adrenal medulla and almost all the NA originates from the sympathetic nervous system.

Because of the very fast rate of removal from the blood the plasma levels under normal conditions are very low It seems safe to accept that at rest human plasma contains less than 1 microgram of free catecholamines per litre The determination of such small amounts of A and NA, even with the best equipment, not only requires large volumes of blood but always involves the element of uncertainty This is one of the several reasons which make catecholamine work with blood an extremely difficult proposition.

For many purposes urinary catecholamine excretion figures are satisfactory or even more meaningful than plasma values in spite of the fact that only a very small fraction of the active amines produced appears in the urine in free form Accepted normal values are 5–10 micrograms of A and 25–50 micrograms of NA per 24 hours In many cases determination of the quantitatively most important common metabolite 3-hydroxy-4-methoxy mandelic acid (HMMA) will add valuable information about the overall catecholamine metabolism. The average normal excretion of HMMA can be taken as 5–10 milligrams per 24 hours. Even an ordinary routine working day imposes many stress conditions on the organism which will be reflected by changes in the secretion and excretion of catecholamines There is a clear cut relationship

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NETHERLANDS SOCIETY FOR PHYSIOLOGY AND PHARMACOLOGY

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The alkaloids erythrophleine and cassaine like ouabain inhibited secretion of cerebrospinal fluid in the cat (VATES *et al.* 1964) and active sodium transport in the toad-bladder (BONTING and CANADY 1964). Contrary to ouabain, the two alkaloids were less effective in inhibiting secretion and transport than in inhibiting Na K activated ATPase activity in choroid plexus and toad bladder. While ouabain inhibited Na transport in toad bladder only from the serosal and not from the mucosal side, erythrophleine inhibited equally on both sides. Apparently erythrophleine, in contrast to ouabain, penetrates cell membranes rapidly owing to its tertiary amine structure and this may explain the discrepancy.

It was concluded that the striking similarity in pharmacological effects of the two groups of substances is the result of their similar effects on the Na K activated ATPase system.

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G. P. M. Horsten and J. E. Winkelman *The effects of transection of the n. opticus immediately after birth on the development of the structure and electrical activity of the retina*

Laboratory of gen. neurophysiology, University of Nijmegen

In an investigation into the centrifugal innervation of the retina, the n. opticus of 45 dogs was transected stereotactically within 4 hours after birth, with the aim to eliminate any influence of these impulses as early as possible.

It became evident that the development of the electrical activity of the retina was not disturbed by this transection.

The histology of the retina of the eyes of these dogs was also discussed.

between the rate of physical and emotional activity and catechol amine excretion For instance during undisturbed sleep NA excretion decreases significantly and there is practically no A excretion The lower HMMA excretion is evidence that not only excretion but production of the active amines is reduced during sleep.

It is obvious that this dependence of catecholamine production and excretion on stress conditions must be kept in mind in all types of experimental work with catecholamines.

S L Bonting *Erythrophleum alkaloids cardiac glycosides and active cation transport*

Dept of Biochemistry University of Nymegen

Erythrophleum alkaloids (*e.g* erythrophleine cassaine) occurring in the bark leaves and seeds of the Erythrophleum family behave pharmacologically almost exactly like the cardiac glycosides (*e.g* digoxin digitoxin ouabain) although chemically the two groups of substances are quite different They have a strong cardiotoxic action which after intravenous injection causes P R prolongation, *bradycardia ectopic rhythm secondary tachycardia* and finally ventricular fibrillation and death In lower doses they have a positive inotropic effect

The cardiac glycosides are known to inhibit sensitively and specifically active cation transport in many tissues This could also be shown for Erythrophleum alkaloids with regard to active potassium uptake in erythrocytes Since the inhibition of cation transport by cardiac glycosides can be explained by the inhibition of the Na K activated ATPase system it was investigated whether a similar relationship might exist for the Erythrophleum alkaloids

Erythrophleine and cassaine in 10^{-4} M concentration strongly inhibited Na K activated ATPase activity in four different tissues, without any effect on the accompanying Mg activated ATPase A slight stimulation occurred at a concentration 1/300 of the half maximal inhibition concentration a phenomenon also observed with the cardiac glycosides. Increasing the potassium concentration in the incubation medium reversed the enzyme inhibition by both groups of substances (BONTING *et al* 1964)

F Kreuzer and J H Schuurmans Stekhoven *Pulmonary O_2 diffusing capacity in the artificially ventilated anaesthetized dog*

Dept of Physiology University of Nijmegen

It has been stated recently that the O_2 diffusion gradient in hypoxia is very small, if not unmeasurable, and that the pulmonary O_2 diffusing capacity is, therefore, very large, both in man and in the dog In order to further substantiate this surprising finding a series of experiments were performed in anaesthetized dogs (pentobarbital) weighing 15–33 kg with artificial ventilation. Both the alveolar arterial O_2 pressure difference ($AaDO_2$) and the arterio-alveolar CO_2 pressure difference ($aADCO_2$) were determined at two levels of hypoxia (0 and 12 % O_2 in the inspiratory gas mixture). The O_2 diffusion gradient was obtained from the total measured $AaDO_2$ by subtracting the term $aADCO_2/R$ (where R = respiratory exchange ratio) as an expression of the distribution component or alveolar dead space contribution of the $AaDO_2$ The results with 9 or 1 % O_2 did not differ significantly and were pooled. The mean $aADCO_2$ was 3 mm Hg (S D = 2.2 mm Hg) the mean $AaDO_2$ 10._ mm Hg (S.D = 4 mm Hg) resulting in an O_2 diffusion gradient of about 8 mm Hg and an O_2 diffusing capacity of about 10 ml O_2/(min × mmHg) Similar results were obtained in another series of experiments in dogs with spontaneous respiration (not reported here). It was concluded that dogs in hypoxia have a well defined O_2 diffusion gradient from which an O_2 diffusing capacity of around 10 ml O_2/(min × mmHg) can be calculated. A similar conclusion was reached for man in a previous study on the O_2 and CO diffusing capacity in normal subjects (KREUZER and VAN LOOKEREN CAMPAGNE, 1965)

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J A J Klijn G P M Horston and J E Winkelman
An objective method for the determination of the oculogram

Laboratory of gen. neurophysiology University of Nymegen

Normally the method of Ardon is used for the determination of the electro-oculogram (E O G) This method necessitates the voluntary co-operation of the test subjects

The optokinetic follow movement of a rotating mirror appeared to give good reproducible E O G values

The mean values, the reproducibility as well as the influence of light and darkness was discussed also in correlation with the blood sugar values

F Krouzor *Physiological meaning of catecholamine excretion in man under various experimental conditions*

Dept of Physiology University of Nymegen

Although the adrenal cortex often receives more attention in connection with the humoral aspects of stress the reactions of the catecholamines also deserve consideration particularly after the development of reliable chemical methods of determination The production of catecholamines occurs in the adrenal medulla (adrenaline) or in the sympathetic nervous system (noradrenaline) or in both The release of adrenaline is influenced mainly by emotional factors and is therefore rather unspecific that of nor adrenaline is regulated by more specific stimuli through the sympathetic nervous system Various cases reported in the literature show the difficulty of distinguishing between different factors such as homeostasis emotions, exercise hypoxia etc. Investigations during a high altitude expedition to Monte Rosa (up to 4500 metres) in 1963 showed a doubling of the concentration of free catecholamines in plasma and urine which was due to an increase of noradrenaline with unchanged adrenaline concentration The free catecholamines as well as the concentration of hydroxy methoxy mandelic acid were also increased during a 90 minute stay in the low pressure chamber at an equivalent altitude but this time the concentration of adrenaline was increased whereas that of noradrenaline was unaltered This difference might be explained by a difference in the emotional conditions.

via dioxyphenylalanine and stored in nervous tissue. The tissue level is kept constant as a result of equilibrium between rate of synthesis and rate of enzymatic oxydation by intracellular monoamine oxydase. The adrenergic neurotransmitters are released upon nervous stimulation adjacent to postsynaptic receptor sites (NA and DA) or into the bloodstream (A). Termination of the neural transmission process is mainly governed by a rapid reuptake process by which released catecholamines are taken back into the neurons. See figure.

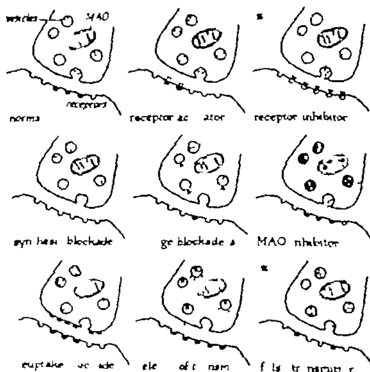


Fig. 1

Schematic representation of the synaptic contact between [illegible] terminal of neurons containing either noradrenaline (NA), dopamine (DA) or 5-hydroxytryptamine (5HT). Drugs may influence neurotransmission at various steps in synthesis, storage, release, breakdown storage and at the receptor level. The symbol § signifies that drugs may act specifically with respect to NA, DA and 5HT. In this respect drugs acting at the other steps d[illegible] not act selectively.

R J van Moerton *Minimum radius of curvature in continuously recorded fractional gas concentration curves in relation to distribution disturbances in the lung*

Department of Lung function Dr van Spanje Hirsel, Groesbeek

One approach for the evaluation of the uneven distribution of ventilation perfusion and diffusion in the lung is the continuous recording of the fractional concentration of gases such as He CO_2 and O_2 during expiration Such an expiratory curve consists first of a continuation of the inspiratory part owing to inspiratory air from the dead space of the airways. Then follows a steeply rising transitional phase which passes over an exponential phase into the flatter alveolar phase in pathological cases almost the whole curve may be exponential On the basis of a simple lung model including one balloon and one tube which may be locally enlarged an exponential function may be derived for the exponential part of the expiratory curve This derivation is based on the assumption that the fractional concentration of an expired gas changes in time proportional to the concentration difference between balloon and tube the proportionality constant is equal to the quotient of gas flow and locally increased airway volume and is the reciprocal value of the time constant (T) It is shown that this time constant may be expressed in terms of the minimum radius of curvature (R_{min}) of the exponential phase. With known gas flow the values of T and P_{min} are a measure of the tube volume locally enlarged in pathological cases like centrilobular emphysema. For cases more complex than this model a study is made of the concentration fronts moving from the alveolar entrances to the mouth where they arrive simultaneously during expiration in normals at rest resulting in $R_{He} = 0$ R_H (after wash in of He) and R_{CO_2} are a measure of disturbance in distribution of ventilation and ventilation perfusion ratio respectively in the lung P_{min} is suggested as a diagnostic criterion for the evaluation of centrilobular emphysema.

J M van Rossum *Pharmacology of adrenergic transmission*

Department of Pharmacology Medical School University of Nijmegen

Adrenergic transmitters dopamine (DA) laevo-noradrenaline (NA) and laevo adrenaline (A) are synthetized from L-tyrosine

INTERFERENCE WITH SYNTHESIS

It is possible to influence adrenergic transmission not only by activation or inactivation of the various types of receptors, but also by influencing synthesis, storage release re-uptake and metabolism of the neurotransmitters.

Laevo-α methylparatyrosine (αMPT) inhibits the enzymatic formation of DOPA from L-tyrosine so that the tissue level of all three catecholamines will be lowered. Obviously those neurons that have a high turnover of catecholamines will first be deficient if synthesis is stopped. αMPT does have an effect in the peripheral nervous system (antihypertensive agent) and in the central nervous system (antipsychotic drug)

A hydrazine analogue of DOPA inhibits the decarboxylation of DOPA to DA. However decarboxylation cannot be blocked completely so that the synthesis of catecholamines is not blocked in this way

Diethyldithiocarbamate and disulfiram inhibit the β-hydroxylation of DA to NA. In addition, this process cannot be inhibited to a sufficient degree in order to stop the synthesis of NA and A. Only in very high doses does disulfiram lower the tissue level of NA. There is a need for a potent β-hydroxylation inhibitor in order to inhibit the synthesis of NA selectively while leaving the DA formation unimpaired.

There are no drugs available yet that block the formation of A from NA

INTERFERENCE WITH ENZYMATIC BREAKDOWN

The intracellular oxydation of catecholamines may be blocked by various drugs characterized as MAO inhibitor as for instance pargyline and nialamide These drugs interfere with the regulation of the tissue level in such a way that the level of catecholamines in the neurons rises by a factor or more It is conceivable that the various MAO inhibitors, although elevating the levels of DA and NA and also of 5-hydroxytryptamine (5HT), influence the tissue level of various amines to a different degree. For example, nialamide causes overt stimulation while iproniazide and pargyline do not, indicating that the former MAO-inhibitor does cause a strong elevation of DA levels.

RECEPTOR SITES

Different effects are produced by the three natural catecholamines as different receptors are involved. The α receptors are typical of noradrenaline and are located adjacent to postsynaptic sympathetic neuron terminals in the vegetative nervous system and to nor adrenergic neurons in the central nervous system (neural transmission) The β receptors are typical of adrenaline and are located in various tissues independent of innervation. Adrenaline reaches the receptors by transport through the bloodstream (humoral transmission) Evidence is available that there are receptors typical of dopamine These dopamine receptors are probably also located adjacent to dopaminergic nerve terminals in peripheral and central nervous system (neural transmission)

RECEPTOR ACTIVATORS

Since the three natural catecholamines are strongly related chemically they may also activate the receptors that are typical of the other amines. By chemical modification however more selective receptor activators can be made than the natural transmitters themselves. Therefore laevo-phenylephrine is a more selective α receptor activator than NA. Laevo-isoprenaline is a more selective β-receptor activator than adrenaline It is expected that also for dopamine receptors more selective activators may be found than dopamine itself These selective receptor activators do not pass the bloodbrain barrier and therefore have only peripheral effect unless they are applied directly to brain structures. The imidazoline compounds naphazoline and tetrahydrozoline are selective α receptor activators which may also have effects in the brain. Dexamphetamine appears to be an activator of dopamine receptors in the brain.

RECEPTOR BLOCKING AGENTS

Neurotransmission of the catecholamines may be interfered with by selective receptor blocking-agents. Piperoxane and yohimbine are relative specific α receptor blockers. Propranolol and pronethalol are specific β receptor blocking agents. Haloperidol and spiramide are relative selective dopamine receptor blocking-agents. All three types of receptor blocking agents may pass the bloodbrain barrier and exert central effects as well as peripheral effects.

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between 76 and 95 %. Despite the marked scattering a clear correlation between muscle mass and water content could be established. The lower the weight of the muscle, the higher was the total amount of water. Parallel to the increase in water an accumulation of sodium per kg wet weight could be observed. These observations suggested that an increase of the extracellular compartment had to be responsible for the elevated water content. As measured by the chloride-space the extracellular compartment indeed proved to increase from ± 13 % in fresh muscle to ± 34 % after captivity. Furthermore these tissue analyses also revealed some intracellular oedema. Since there seemed to exist a relationship between water content or extracellular space and muscle mass we tried to get information about the radius of the muscle fibre. For this reason we calculated the fibre diameter on the basis of the potassium content. From these calculations it followed that the mean diameter of 80 µ decreased to 34 µ after captivity. To test these calculations planimetric studies as well as function-tests (twitch) were performed on both fresh and old frogs. By these two methods the same diameter could be established.

In conclusion we may say that all changes observed are in favour of atrophic alterations. They provide a basis for under standing why extreme variations in extracellular fluid compartment are so often found, if the Sartorius muscle is used for study. It could be shown that, to exclude atrophic muscles for experimental work, a simple water-determination on the control muscle would suffice.

W. A. Smit, *Zoological Institute, University of Amsterdam*,
G. Becht and A. M. Th. Beenakkers, *Zoological Institute Catholic University of Nijmegen*

Correlations between structure and function in insect muscles

Correlations were found between (a) the colour and the number of sarcosomes, (b) some enzymes of the energy producing pathway and (c) the staying power.

Mechanical functions of muscle are graded by internal properties as well as neuromuscular control.

INTERFERENCE WITH STORAGE AND RELEASE

Reserpine and tetrabenazine reduce tissue levels by damaging the storage vesicles in the cell Depletion of amines however may not be complete since synthesis is not impaired These drugs *impair neurotransmission by depletion of all NA DA and 5TH* both in the central and peripheral system

Tyramine releases NA from neurons and so causes an indirect α-sympathomimetic effect. Amphetamine does act similarly in the peripheral nervous system on NA neurons In the central nervous system it may also release NA from NA neurons but also directly activate dopamine receptors.

Specific dopamine releasers are not known although there is evidence that benzphetamine and cocaine may release dopamine in the central nervous system

FALSE TRANSMITTERS

Methyldopa is converted into methyldopamine and methylnor adrenaline which act as releasers and to a certain extent as false transmitters, since they take over the role of noradrenaline while being less efficient receptor activators.

INTERFERENCE WITH UPTAKE

Imipramine desipramine protriptyline and other thymoleptic drugs inhibit the re-uptake of NA but also of DA in noradrenergic and dopaminergic neurons respectively In this way they increase the concentration of the neurotransmitters near the receptors and facilitate the transmission process

Many substances are not selective since they influence the transmission process in more than one way It is obvious that selectively acting drugs should be used as tools in investigations on the nervous system

J F G Slegers *Water and electrolyte abnormalities of the atrophic Sartorius muscle of the frog*

Department of Physiology University of Nymegen

Studying Sartorius muscles of frogs kept in the animal house during winter it was found that the total water content varied

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NETHERLANDS SOCIETY FOR PHYSIOLOGY AND PHARMACOLOGY

PROCEEDINGS 104TH MEETING GRONINGEN NOVEMBER 19 1966

A A J Verhofstad *About morphological research methods for catecholamines and their distribution in mammalian tissues*

Department of Anatomy University of Nymegen

In this lecture the histochemical techniques for catecholamines was reviewed From each method were explained reaction mechanism specificity sensitiveness, and the degree of cellular localization. After that a survey was given of the distribution found in mammalian tissues

In conclusion the meaning of electron microscopic and autohistoradiographic methods was briefly discussed

A Hrbek *Somatosensory EEG responses in newborn infants accompanied with monosynaptic reflexes*

Dept of Developmental Neurology University of Groningen

Using averaging techniques, cortical evoked potentials accompanying monosynaptic reflexes (knee jerk, biceps- triceps and lip-reflex) in infants were discovered. Seven infants, six newborns and one in the age of five weeks, were examined.

The observed evoked responses may be divided into two components an early primary component most pronounced in the corresponding projection area of the contralateral side and a late component occurring symmetrically on both hemispheres and reaching maximum around the vertex. The peak latency of the first deflection is on the average about 50 msec during the first ten days of life This value decreases during the next days and weeks.

The responses behave differently in the two phases of sleep In rapid eye movement phase" the amplitude of the primary component is increased while the amplitude of the late part is decreased in relation to the response pattern in the slow wave sleep.

Comparative examinations carried out in three adults show that there are no important differences in the shape of the response between newborn infants and adults. Only the time-course in adults is much faster

Comparison with previously studied visual evoked potentials (HRBEK and MAREŠ 1964 HRBEK *et al.* 1966) gives evidence for a greater maturity of the somatosensory afferent system in newborn infants.

It is supposed that the described evoked responses are proprioceptive in origin.

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H D Coster *The design of a very fast tonometer for screening purposes*

Laboratory of Physics University of Groningen

Tonometers measure the intra-ocular pressure from the outside by deforming the cornea The relation between force and deformation gives information on the pressure as far as the deformation energy is carried hydrostatically to the deeper parts of the eye

The existing tonometers are static instruments i e during measurement all parts of instrument and eye are at rest. For screening purposes however they are too time-consuming

In recent years the late Professor H M Dekking of the Ophthalmological Institute of Grongingen University developed a dynamic method based on the idea that the relationship between force and deformation is reflected in the shape of the time – velocity curve of an object that collides with the cornea.

The present author has assisted in the choice of optimum parameters Shape mass and speed of the colliding hammer are the three main degrees of designing freedom In order to minimize the energy spent in deforming the cornea (which is a function of its elastic properties and not of the intra-ocular pressure), the radius of curvature of the colliding hammer surface should not be smaller than that of the cornea which as it consists mainly of fibrous tissue will develop high stresses when lengthened but not when shortened We have accordingly chosen a convex spherical hammer tip with a radius of curvature equal to that of the cornea. The collision assumes the character of half a period of a harmonic oscillation which makes it attractive to use the collision time as the measured quantity as it is independent of the hammer's initial speed. The hammer bounces back before the subject can react and anaesthesia is superfluous

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and found for pikes of 4.7 cm and 97 cm values of 360 cm and 310 cm respectively

The amplitude of the top of hairs will then be no more than 0.04 Å. This is in the same order of magnitude as the minimum displacement of the hairs in the organ of Corti in the human ear

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J W Kuiper *The directional sensitivity of single retinula cells in the compound eye of the blowfly (Calliphora Erythrocephala)*

Department of Biophysics Laboratory of Physics University of Groningen

The directional sensitivity φ of a single retinula cell is determined by the diameter of the receptor part of the cell and by the image formed by the refractive system Because of refraction and diffraction of the small lens there is no sharp focal point, but a focal line which extends along the optical axis (Kuiper, 1967)

The directional sensitivity calculated by using the geometric parameters and the refractive indices involved is in accordance with the electrophysiological findings of Washizu *et al.* (1964).

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Washizu Y D Burkhardt and P Streck, Z. vergl. Physiol. 48; 413–428 (1964)

H G Lenard *Eye movements in sleeping newborn infants*

Dept of Developmental Neurology University Hospital, Groningen

Rapid eye movements (REM) during the so-called "REM phase" (Aserinsky and Kleitman 1953) or "paradoxical phase (Jouvet 1965) of sleep have been recorded electro-oculographically in the course of polygraphic investigations in 17 full term healthy newborn infants. REMs are present continually but in an irregularly varying amount during an epoch of REM-sleep. Parallel recordings with time constants of 0.1 and 1 0 showed that REMs are always

J. H. ten Kate *The oculo-vestibular reflex mechanism of the pike (Esox Lucius) during growth*

Department of Biophysics, Laboratory of Physics, University of Groningen

The dimensions of the labyrinth undergo notable changes during growth. The threshold of the oculo vestibular reflex however is independent of the length of the pike, as we have established experimentally for horizontal angular acceleration. We accordingly pose the question how this is possible since the mechanism of the eye movement behaves linearly near threshold and is independent of the size of the pike.

The threshold of the senso epithelium is a function of the area A enclosed by the canal, the length l_1 and the radius r_0 of the narrow part of the canal, the viscosity η and the density ρ of the endolymph and the cross-section O of the ampulla. With the aid of the differential equation proposed by Hl. DE VRIES (1956) we derive

$$= \frac{A\rho}{4\eta l_1} \frac{\Pi r_0^4 \gamma}{O_a} \quad (1)$$

γ is the angular velocity of the sinusoidally moving animal.

We determined the dimensions of the labyrinth in 40 pikes, varying in body length (l in cm) between 4.7 and 97 cm.

We obtained $A = 0.065\ l^{1.76}$ mm^2 $\quad l_1 = 0.50\ l^{0.81}$ mm

$O_a = 0.036\ l^{0.84}$ mm^2 $\quad r = 0.065\ l^{0.25}$ mm

We also measured $\eta = 0.0125$ Poise, $\rho = 1.007$ g/cm^3 and $\gamma = 0°$/sec.

With (1) we find

$$= 14.1\ l^{0.97}\ A \quad (2)$$

As is still dependent on l we determined the height h_c of the cupula

$$h = 0.20\ l^{0.48} \text{ mm}$$

Now $/h^2 = 3.5 \times 10^{-5}\ l^{0.01}$ is practically independent of the body length. One possible conclusion is that the stimulation of the organ *is mediated through the bending of the cupula*. We have calculated its radius of curvature resulting at threshold stimulation

risetime (25 μsec) for both eyes. The left flash, however was delayed with respect to the right one by a time Δt

We determined the threshold value Δt for which a positive recognition of the existence of a delay between the two flashes was given with a probability P and found $P = 60$ % for $\Delta t = 1$ msec, and $P = 100$ % for $\Delta t = 20$ msec. Plotting P versus Δt we obtained a curve that in the range of 1 msec $< \Delta t < 17$ msec can be nicely approximated by the linear relationship $P = 1.9\, \Delta t + 58$ %.

This result should enable us to determine whether or not there are significant differences in coding between stimuli whose product of intensity and duration is constant (reciprocity relation) but are unequal in duration If these coding-differences should result in a change in perception-delay of a few msecs

In the second series of experiments we accordingly changed one of the flashes (the left one) in such a way that the product of intensity and duration was kept constant, and chose for the duration $T_L = 4440$ μsec. ($= 12$ times T_R) and $T_L = 8880$ μsec ($= 4$ times T_R) Plotting the resulting threshold values Δt against P we found two curves that are similar to the one obtained with identical flashes except for a displacement to the left by an amount of 4 msec and 8 msec respectively

We conclude therefore that there does indeed exist a different coding for unequal flashes within the reciprocity relation.

C J den Otter *Specificities of the sense cells of taste hairs of Calliphora erythrocephala Mg*

Zoological Laboratory University of Groningen Haren

To ascertain the nature of the cells innervating taste hairs of the bluebottle Calliphora erythrocephala Mg. we recorded the impulses generated in these hairs when applying water and different concentrations of sodium chloride and sucrose Moreover we stimulated taste hairs with 0.5 M solutions of different chlorides and potassium salts.

The numbers of cells active during the stimulations were determined by making frequency distributions of the amplitudes of the impulses

When stimulating with salt one two or three cells became active depending on the salt concentration and on the location

superimposed on slow rolling movements Interval histograms of REMs have negative exponential distributions which seem to be the result of several random processes. Interval histograms of eye movements of awake babies are more regular and contain less longer intervals In contrast to observations in adults (DEMENT 1964) there are no eye movements at all during quiet sleep in newborns. Synchronously with transient increases in the amount of REMs there is an increase in frequency and irregularity of respiration a depression of monosynaptic reflexes and an increase in the amount of small muscular twitches There are no significant changes in heart rate and no changes in the power density spectrum of the parieto-occipital EEG dependent on the amount of REMs.

BIZZI *et al* (1964) found an increased discharge rate during REMs in the medial and descending vestibular nuclei POMPEIANO and MORRISON (1965) proved that bilateral intactness of these structures is indispensable for the occurrence of REMs. Statistical analysis suggests that the eye movements during REM sleep represent a complex random process One might hypothesize an increase of random noise in the vestibular nuclei during REM-sleep.

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POMPEIANO O and A. R. MORRISON Arch. Ital. Biol. 103; 500–505 (1965).

H A K Mastebroek and J W Kuiper *The time resolving power in binocular vision*

Department of Biophysics Laboratory of Physics University of Groningen

In the first series of experiments the subject's right and left eye have been stimulated separately (in the binocularly fused field of vision) with rectangular flashes of bright white light that were identical in intensity duration ($T_R = T_L = 3$ 0 μsec) and

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Department of Pediatrics, University of Nijmegen

THE BEHAVIOUR OF EVANS BLUE (AZO-DYE T 1824) IN THE BODY AFTER INTRAVENOUS INJECTION [1]

BY

G. B. A. STOELINGA AND P. J. J. VAN MUNSTER

INTRODUCTION

Evans blue is frequently used to determine the plasma volume. The substance combines specifically and firmly with albumin as long as the combining capacity of albumin for the dye is not exceeded. This is why it was believed that the plasma disappearance curve of T 1824 would yield further data about albumin catabolism. The literature shows divergence of opinion on this disappearance curve. Therefore in the present investigation we re-studied this curve in human subjects over a considerably longer period than most investigators did, using a new method to determine the Evans blue.

About the distribution of T 1824 through the body its excretion and break-down little is known.

It has been established that the dye disappears more rapidly from the blood than does ^{131}J albumin. This made LE VEEN and FISHMAN (1947) suggest that in the body the dye is released from the albumin without the albumin being metabolized. To investigate the rapid disappearance of Evans blue from the blood we studied the total excretion of the dye and whether somewhere in the body T 1824 is accumulated or broken down.

MATERIALS AND METHODS

THE GALLBLADDER FISTULA

Via a laparotomy a gallbladder fistula was made in dogs according to MARKOWITZ (1954). The bile was caught in a rubber bag and collected once in 4 hours through a three-way tap.

[1] Supported by a grant from the Organisation for Health Research T.N.O.

of the hair (DEN OTTER in press) Two groups of cells could be distinguished i.e. salt cells and non salt cells. The salt cells were mainly responsive to cations but presumably also to anions (DEN OTTER and VAN DER POEL, 1965) The non salt cells could be subdivided into more or less sensitive water receptors and cells which till now have an unknown function

The responses we obtained to different sucrose concentrations showed activity of two cells (DEN OTTER and VAN DER STARRE, in press) The responses of one of these cells were clearly positively correlated with the changes in sugar concentration the impulses of the other cell showed a tendency to increase their frequency when the sugar concentration was lowered In by far the most cases water also evoked activity in two cells (DEN OTTER and VAN DER STARRE in press) In about 10 % of the stimulations impulses of one cell only have been recorded

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DETERMINATION OF EVANS BLUE IN THE BILE

When trying to determine the amount of Evans blue in bile according to the method as described for plasma we were faced with the difficulty that to a certain extent bilirubin too, is adsorbed on cellulose. This prevents calculation of the quantity of T 1824 from the difference in optical density before and after adsorption. For this reason we eluted the dye quantitatively from the cellulose column using dimethylformamide (DMF). We preferred DMF to 50% acetone, a solvent which was recommended by ALLEN (1951 1953), because in our experience acetone does not elute the adsorbed dye quantitatively from the column. This results in erroneous values especially in the low concentration range.

DMF however also partly removes the adsorbed bilirubin. By measuring the optical density of the eluate at 620 mμ and at 450 mμ (the adsorption maxima of Evans blue and of bilirubin respectively) a correction for the co-eluted bilirubin can be applied as follows

$$O.D._{corrected} = O.D._{620} - 0.04 \times O.D._{450} + 0.004 \times O.D._{[illegible]}$$

Oxidation of bilirubin is prevented by adding ascorbic acid to the bile

Procedure

To 10 ml bile 10 ml of a twice-diluted solution of concentrated Rogypon TZ and 20 ml of a 200 mg/100 ml solution of ascorbic acid in 0,9 % NaCl are added This mixture is centrifuged for 10 min at 15 000 × g.

A glass tube 5 cm in length and 0 5 cm in internal diameter is loosely packed with Whatman nr 1 paper powder By a length of para-rubber tubing previously immersed in DMF for 4 hours to extract any traces of dye, this column is fitted to a funnel.

A known quantity of the bile mixture is filtered and the column is washed with [illegible] ml 0 9% NaCl which is sufficient to remove the not adsorbed excess of bilirubin. Next the glass tube is inverted and the column is eluted with 1 ml DMF 1 ml 0 9% NaCl 1 ml DMF 1 ml 0,9% NaCl and 1 ml DMF respectively The volume is made up to 5 ml with 0 9 % NaCl. After reading the optical density at 450 and 620 mμ the correction for bilirubin is applied. Standards are

PURIFICATION OF EVANS BLUE

Commercial preparations of Evans blue often contain a red impurity (GREGERSEN AND GIBSON 1937, SCHWARTZKOPF 1960), which in our Merck and Geigy batches amounted to 4–5%. In order to ensure reliable data the dye was first purified. An aqueous solution of the impure Evans blue was treated with concentrated sodium acetate. This procedure was repeated until the dye was chromatographically pure (BLATT Organic Synthesis 1944).

DETERMINATION OF EVANS BLUE IN THE PLASMA

In general the concentration of Evans blue in the plasma is determined spectrophotometrically, a correction being necessary for the plasma's own colour. As this colour is not constant, however, plasma samples collected at the beginning of some long term investigation should not be used as blanks over the entire period. CONOLLY and WOOD (1954) arrived at a correction based on optical density (O.D.) measurements at various wave-lengths. Such a correction is less reliable though and may result in considerable errors, especially at low dye concentrations. For this reason we used the method developed by WYERS and VAN MUNSTER (1961). In this method the "blank" value of each plasma sample is measured by eliminating the Evans blue from the plasma to be investigated. To achieve this the Evans blue-albumin bond is dissolved by a detergent, after which the free dye in the presence of NaCl is adsorbed on a loosely packed column of Whatman nr. 1 cellulose powder placed inside the stem of a 4 cm funnel.

Procedure

3 ml of plasma is diluted with 6 ml of 0.9% NaCl and 3 ml of a 20% solution of Rogypon TZ [1] (a domestic detergent). The optical density of this mixture is measured *before* and after the adsorption of the dye on cellulose at 620 mμ in 4 cm microcells using a Unicam SP 500 spectrophotometer. From the difference in optical densities the Evans blue concentration is calculated by comparison with a standard curve. This standard curve is perfectly linear.

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1) Obtained from Rogier-Bosman, Rotterdam, The Netherlands.

As was to be expected, the sterile faeces contained a considerable amount of undigested cellulose to which a large part of the dye remained adsorbed. After repeated extraction with DMF finally 80–90% of added quantities of Evans blue were recovered

Procedure

10 grams of faeces are ground in a mortar together with 40 ml DMF and quartz sand. After centrifuging at 15 000 × g the sediment is extracted with, respectively 20 ml DMF 20 ml 0 9% NaCl, 20 ml DMF 20 ml, 0 9% NaCl and 20 ml DMF

The total amount of extract is desiccated *in vacuo* (0 1 mm Hg at 40 C). The residue is dissolved in a known quantity of a 5% Rogypon solution. After centrifuging at 15000 × g the determination of the Evans blue is carried out according to the method as described for bile

EXPERIMENTS AND RESULTS

In 8 healthy men, who had fasted for 10 hours 0,5 mg Evans blue per kg body weight was injected intravenously According to RAWSON (1943) ALLEN and ORAHOVATS (1950) and SCHWARTZKOPF and HÖLZER (1960) this dosage does not exceed the binding capacity of albumin for Evans blue Ten minutes after injection the plasma dye concentration was determined this value was considered to be the initial value. At first daily but afterwards twice a week, the determination was repeated and thus we followed the disappearance of the dye from the plasma for about 65 days. The data for each individual were plotted semilogarithmically against the time. The curves showed all the same shape (Fig 1a and Fig 1b) After the tenth day three randomly chosen curves were subjected to a regression analysis. A straight line and a parabola were fitted to these curves. The reduction of the residual variance obtained by use of the parabola instead of the straight line was tested against the residual variance about the parabola, using the conventional F test (SNEDECOR, 1956). The results appeared to be highly significant in favour of the parabola. Therefore we concluded that the linear fit was insufficient.

In the same way in 8 normal dogs (weight c. 20 kg) plasma disappearance curves were plotted over a fortnightly period after

obtained by adding known quantities of Evans blue to the same medium

Recovery test

To 5 ml of bile diluted as described known quantities of Evans blue were added and the recovery was calculated

Added	Recovered	Percentage
2.00 μg	1 97 μg	98.5
4 00 μg	3 9- μg	98
8.00 μg	7 72 μg	96 5

DETERMINATION OF EVANS BLUE IN ORGANS

In this investigation the animals were bled to death under penthothal anaesthesia A weighed-out part of an organ was cut up and ground with a known quantity of 0 9% NaCl in a Bühler homogenizer at 50 000 rpm After the tissue pulp had been diluted with an equal volume of a 50% Rogypon solution and 2 volumes of 0 9% NaCl the mixture was centrifuged at 15 000×g In the *supernatant the Evans blue was determined* by the method as described for bile Next, the amount of Evans blue per gram (wet weight) was calculated In a few cases the sediment contained shreds of tissue which still showed traces of dye Accordingly in such cases the determination would yield values that were slightly too low

DETERMINATION OF EVANS BLUE IN FAECES

Evans blue is decoloured by the intestinal flora. Therefore when the occurrence of T 1824 in faeces was investigated the contents of the dogs intestines were sterilized For this purpose they received *3 days before* the administration of the dye and during the experimental period every 6 *hours* 2 tablets of Nobacetin[1]) *orally* These antibiotics were effective in suppressing the intestinal flora for 8–10 days. Evans blue solutions incubated with faeces obtained during this period were not decoloured

[1]) 1 tablet Nebacetin forte (Lundbeck) contains 165 mg Neomycine B base and 12.500 U Bacitracine.

As was to be expected, the sterile faeces contained a considerable amount of undigested cellulose to which a large part of the dye remained adsorbed. After repeated extraction with DMF finally 80–90% of added quantities of Evans blue were recovered

Procedure

10 grams of faeces are ground in a mortar together with 40 ml DMF and quartz sand. After centrifuging at 15 000 × g the sediment is extracted with, respectively 20 ml DMF 20 ml 0 9% NaCl, 20 ml DMF 20 ml, 0,9% NaCl and 20 ml DMF

The total amount of extract is desiccated *in vacuo* (0 1 mm Hg at 40 C) The residue is dissolved in a known quantity of a 5% Rogypon solution. After centrifuging at 15000 × g the determination of the Evans blue is carried out according to the method as described for bile.

EXPERIMENTS AND RESULTS

In 6 healthy men, who had fasted for 10 hours 0 5 mg Evans blue per kg body weight was injected intravenously According to RAWSON (1943), ALLEN and ORAHOVATS (1950) and SCHWARTZKOPF and HÖLZER (1960) this dosage does not exceed the binding capacity of albumin for Evans blue Ten minutes after injection the plasma dye concentration was determined this value was considered to be the initial value At first daily but afterwards twice a week, the determination was repeated and thus we followed the disappearance of the dye from the plasma for about 65 days. The data for each individual were plotted semilogarithmically against the time. The curves showed all the same shape (Fig. 1a and Fig. 1b) After the tenth day three randomly chosen curves were subjected to a regression analysis. A straight line and a parabola were fitted to these curves. The reduction of the residual variance obtained by use of the parabola instead of the straight line was tested against the residual variance about the parabola, using the conventional F test (SNEDECOR, 1956) The results appeared to be highly significant in favour of the parabola. Therefore we concluded that the linear fit was insufficient.

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Recovery test

To 5 ml of bile diluted as described known quantities of Evans blue were added and the recovery was calculated

Added	Recovered	Percentage
2.00 µg	1.97 µg	98.5
4.00 µg	3.9* µg	98
8.00 µg	7 µg	96.5

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[1]) 1 tablet Nebacetin forte (Lundbeck) contains 165 mg Neomycine B base and 1*.500 U Bacitracine.

As was to be expected, the sterile faeces contained a considerable amount of undigested cellulose to which a large part of the dye remained adsorbed. After repeated extraction with DMF finally 60–90% of added quantities of Evans blue were recovered

Procedure

10 grams of faeces are ground in a mortar together with 40 ml DMF and quartz sand. After centrifuging at 15 000 × g the sediment is extracted with, respectively 20 ml DMF 20 ml 0 9% NaCl 20 ml DMF 20 ml, 0 9% NaCl and 20 ml DMF

The total amount of extract is desiccated *in vacuo* (0 1 mm Hg at 40° C). The residue is dissolved in a known quantity of a 5% Rogypon solution. After centrifuging at 15000 × g the determination of the Evans blue is carried out according to the method as described for bile

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In the same way in 8 normal dogs (weight c. 20 kg) plasma disappearance curves were plotted over a fortnightly period after

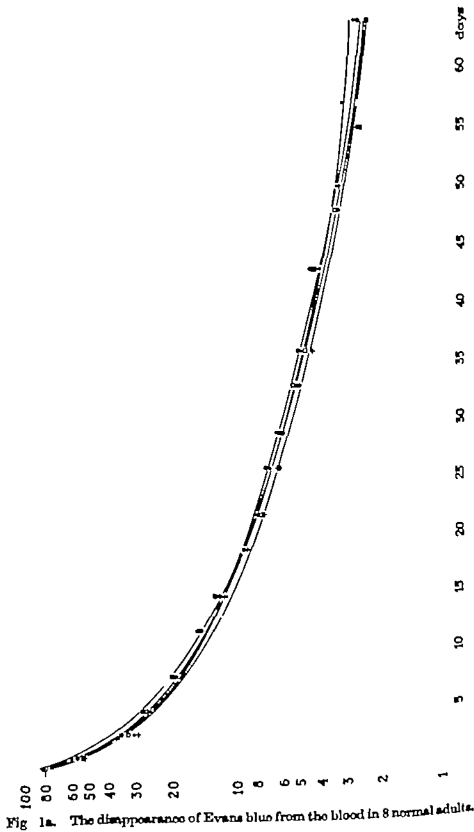


Fig 1a. The disappearance of Evans blue from the blood in 8 normal adults.

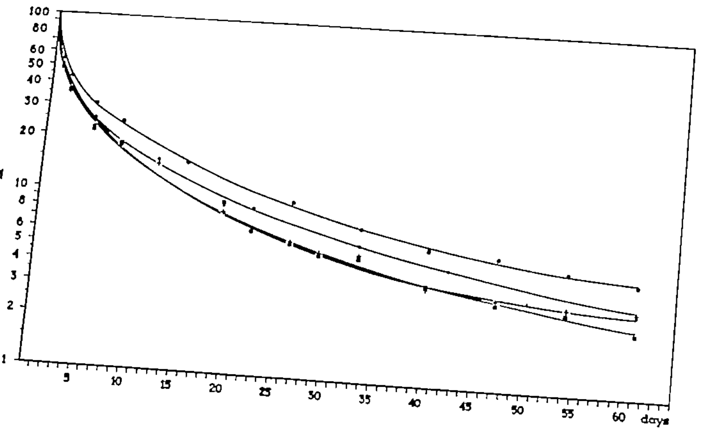

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Fig 1b.

intravenous injection of 2–3 mg Evans blue per kg body weight. This dosage did not exceed the binding capacity of the albumin either The individual curves of the dogs are presented in Fig 2.

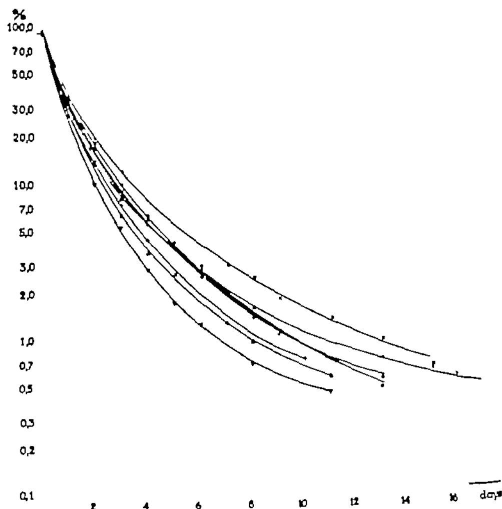


Fig 2. The disappearance of Evans blue from the blood in 8 normal dogs.

From these curves the arithmetic average was calculated resulting in curve A (Fig 3) In dogs the disappearance of the dye from the plasma occurs at a considerably more rapid rate than in man which confirms the findings of SEAR *et al* (1953) and of ALLEN and GREGERSEN (1953)

In six other dogs a gallbladder fistula was made and the bile produced was collected. The quantities varied between 100–200 ml

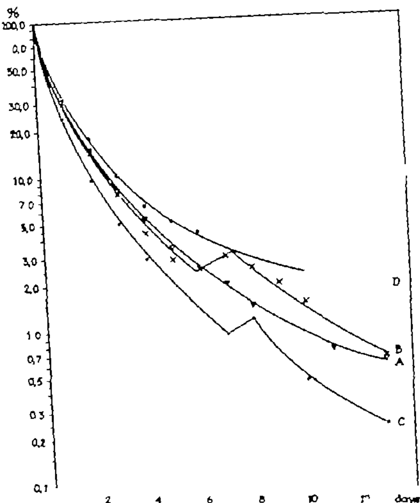


Fig. 3. The disappearance of Evans blue from the blood in dogs. A: The arithmetic average of the 6 normal curves plotted in Fig. 2. B and C: In these curves the sharp break indicates the moment when the gallbladder-fistula became obstructed. After one day the curve follows its initial course. D: Disappearance curve in dog in which the d. choledochus was operatively ligated.

a day being fairly constant for each dog. After intravenous injection of Evans blue, disappearance curves were obtained; these curves

were the same as those of normal dogs. Besides the daily excretion of the dye with the bile was determined In Fig 4 these values are plotted as percentages of the injected dose.

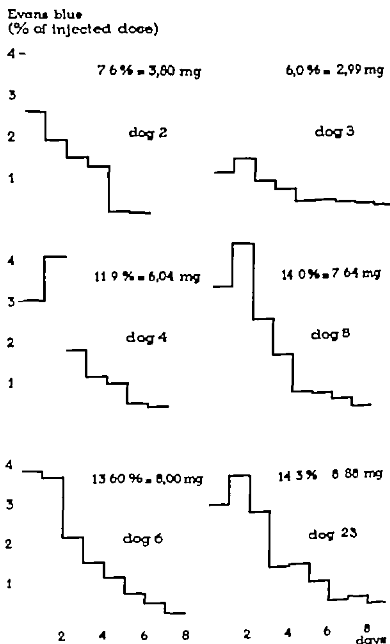


Fig 4. Daily excretion of Evans blue in the bile after intravenous injection in dogs plotted as percentages of the injected dose.

Analysing the plasma disappearance curve, WYERS and VAN MUNSTER (1962) assumed that every day a constant percentage of the average amount of dye circulating in the blood is excreted in the bile. We therefore calculated the percentage of the daily amount of T 1824 excreted in respect of the quantity circulating in the blood on that particular day. As is shown in Table 1 these

TABLE 1

Amount of Evans blue daily excreted in the bile in percentages of the average quantity of dye circulating in the blood on that particular day. The amount of dye is calculated from the individual disappearance curves

Dog	Days after intravenous injection								
	1	2	3	4	5	6	7	8	9
2	3,2 %	10 1 %	11,6 %	12,8%	27 4%	18,8%			
3	2 48%	8,85%	8,64%	12,6%	10,9%	16,0%	18 4%	24 0%	
4	10 1 %	27 6 %	26,3 %	29,8%	40,9%	40,0%	25,2%		
5	4,8 %	22,4 %	20,3 %	35,0%	32,9%	34,0%	37 0%	30,0%	
6	6,1 %	24,8 %	22,4 %	44,0%	58,0%	61 0%	46,0%	27,0%	
12	6,0 %	18,8 %	39 4 %	24,0%	39 0%	39 0%	26,1%	42,0%	30,0%

percentages are not constant however. The total excretion of T 18.4 in the bile after intravenous injection appeared to be rather small, maximally not more than some 15% of the quantity injected was recovered (Fig. 4). In two dogs the gallbladder fistula became obstructed in the course of the investigation. This resulted in but a brief rise of the plasma Evans blue level, after which the disappearance curve resumed its original course (Fig. 3B, 3C). Also, after ligation of the bile duct to prevent the excretion of dye via the biliary ducts the disappearance curve deviated only slightly from the normal one (Fig. 3D).

After intravenous injection of Evans blue in dogs the dye concentration in various organs was determined. It turned out to be considerable in the liver, kidneys, spleen and in the lymph nodes (Table). In the lungs, heart, skeletal muscles, intestinal mucosa and in subcutaneous tissue few if any traces of the dye were seen.

TABLE 2

Recovered amount of Evans blue in some organs of the dog after intravenous infection of this dye.

dog	mg infected		number of days after b. B injection	Liver mg	Liver mg/g	Liver %	Kidney mg	Kidney mg/g	Kidney %	Spleen mg	Spleen mg/g	Lymph nodes mesenterio mg/g	Lymph nodes arm. pit mg/g	Lymph nodes average of others mg/g
17	56,2	No bile fistula	8	14,3	0 0303	25 5	2,7	0 0281	4 8	—	—	0 0195	0,0128	0 0130
11	48	No bile fistula	14	12,..5	0 0 15	25	1,28	0 010	2,7	0,230	0 0046	0 0300	0,01°3	0 0082
13	49	No bile fistula	14	12,3	0 0300	2.,	1,30	0 019	2,8	0 420	0 0084	0 0250	0,0114	0 008
15	56,5	No bile fistula	19	5 1	0 0119	9	1 7	0 017	3 0	—	—	0,0180	0 012	0 008
10	47 5	No bile fistula	21	12 7	0 023	27	1 76	0 022	3 7	0,368	0 0046			
12	50	No bile fistula	32	6,08	0 0102	12	0 94	0 0115	1 9	0,282	0 0047			
16	50	No bile fistula	38	3 10	0 0033	6,5	0 84	0 011	1 7	0 195	0 0027	0 0150	0 006	0 007
6		Fistula performed	12	17 6	0 0355	30	1 87	0 0183	3,2	0 466	0 0102	0,0117	0 0035	0 0035
7	55	Fistula performed	12	15 6	0 0296	28	2,35	0 0287	4,3	0 600	0 012			
5	54	Fistula performed	19	13 8	0 0244	25	2,24	0 018	4 15	0 100	0 0035			
8	43,5	Fistula blocked	5	13 85	0 036	32	1 49	0 0214	3 4	0,251	0 012	0 0465	0 0235	0 016
9	51	Fistula blocked	14	12 8	0 015	25	2 04	0 0136	4 0	0,270	0 0046			
14	74	Ductus Choledochus ligated	6	25,9	0 039	35	4 51	0 041	6 1	—	—	0 0303	0 0075	0 025
19	50,5	Ductus Choledochus ligated	6	14,2	0 019	28	2 06	0 026	3,85	—	—	0 022	0 017	0 025

mg = amount dye in mg
mg/g = mg of dye per g tissue (wet weight)
% = percentage of infected dose.

In three dogs both the quantity of T 1824 in the organs and the quantity excreted in the bile was determined. In total 36–47% of the injected dose was recovered (Table 3)

We investigated whether after intravenous injection of Evans blue the dye is excreted in the faeces. For this purpose we first tested whether the dye is absorbed in the intestines. After sterilisation of the intestinal tract with Nebacetin a dog was given 200 mg of Evans blue orally Whereas in the faeces 165 mg was recovered, no dye could be traced in the plasma and in the various organs. In another dog, which had received 10 mg Evans blue *per os*, 8,63 mg was recovered. As mentioned before *in vitro* determinations of the amount of dye in faeces gave a recovery of 80–90%.

Accordingly it is our view that there is hardly any absorption of the dye in the intestines.

Next, in two dogs the bile duct was ligated and a third was fitted with a gallbladder fistula, after which these three dogs received an injection of 3 mg Evans blue per kg body weight. Only over the first five days after this injection measurable quantities of dye were found in the faeces the total quantity excreted was respectively 2.6% 2.93% and 1 74% of the injected dose.

DISCUSSION

RAWSON (1943) ALLEN and ORAHOVATS (1950) and SCHWARTZKOPF and HÖLZER (1960) calculated that 1 albumin molecule is capable to bind 11–13 molecules of the dye. ALLEN and ORAHOVATS (1951) stated that this bond is so firm that in normal plasma there will occur 1 free dye ion as against 1000 albumin-bound molecules.

The distributions of Evans blue over the intravascular space and its disappearance during the first hour after intravenous injection were reported to be equal to that of ^{131}J albumin by CRISPELL *et al* (1950) SEAR *et al* (1953) and SCHULTZ *et al.* (1953). Studies of LE VEEN and FISHMAN (1947) CONOLLY and WOOD (1954), WYERS and VAN MUNSTER (1961) and GARROW and WATERLOW (1959) showed however that after the first hour the dye disappears from the blood much more rapidly Hence LE VEEN and FISHMAN (1947) and GARROW and WATERLOW (1959) inferred that although the bond between Evans blue and albumin is very firm, Evans blue is not a genuine tracer for albumin. The rapid

TABLE 3

Total quantity of Evans blue recovered in a few organs and in the bile 12–19 days after intravenous injection of this dye in dogs

Dog	Injected dose		Days after i.v Evans blue injection	mg of dye in:				Total
	Total	mg/kg body weight		Liver	Kidney	Spleen	Bile	
5	54 1 mg	2,32	19	13,8	2,24	0 10	7 64	23 78 mg = 44%
6	59 0 mg	2,50	12	17 6	1 87	0 46	8 00	27 93 mg = 47%
7	54,8 mg	2 67	12	15 6	2,33	0 66	1,25	19,86 mg = 36%

% = *percentage of the injected dose.*

disappearance of the dye from the blood might be explained if it could be shown that somewhere in the body a considerable amount of Evans blue is excreted accumulated or broken down. This problem was studied by us in dogs.

MILLER (1947) found that Evans blue is excreted in the bile over the first hour the excretion turned out to be on an average 4% of the quantity injected. After a latent period the concentration in the bile decreased proportionally with the blood concentrations, but at a lower level.

In our experiments we found the total excretion of T 18 4 in the bile after intravenous injection to be rather small, maximally not more than some 15% of the quantity injected was recovered (Fig 4). Moreover we showed that this excretion over the second day when the concentration in the plasma has already dropped considerably is as high or even higher than over the first twenty four hours (Fig 4). Thus we may speak of a certain tardiness in the excretion through the liver

Obstruction of the bile duct resulted in only a slight deviation of the plasma disappearance curve in comparison with the normal one (Fig 3B C D).

These observations point to the fact that the excretion of dye in the bile has no considerable effect on the course of the disappearance curve In our animals which were free from albuminuria, there was no excretion of dye in the urine We succeeded in demonstrating that after intravenous injection of Evans blue this dye was excreted in the faeces. But this excretion was no more than a slight percentage of the total injected dose (1 74–3 6%) for our investigation it was of little interest. It does support, however the assumption of HOLMAN *et al* (1934), WETTERLOEF *et al* (1960) and of BARANDUN *et al* (1960) that excretion of serum albumin in the intestines is part of the normal protein catabolism, for the only possibility for Evans blue to enter the intestinal tract is when bound to albumin. Because as we showed there is no resorption of dye in the intestines the excretion of Evans blue in the faeces, consequently is a measure for the excretion of albumin in the intestines. As, however the determination of the dye in the faeces is not sufficiently exact it was not possible to arrive at a quantitative calculation of the intestinal albumin catabolism.

As the disappearance of dye from blood cannot only be explained

TABLE 3

Total quantity of Evans blue recovered in a few organs and in the bile 12–19 days after intravenous injection of this dye in dogs.

Dog	Injected dose		Days after i v Evans blue injection	mg of dye in				Total
	Total	mg/kg body weight		Liver	Kidney	Spleen	Bile	
5	54,1 mg	2,32	19	13,8	2,24	0 10	7 64	23,78 mg = 44%
6	59 0 mg	2,50	12	17 6	1,87	0 46	8,00	27 93 mg = 47%
7	54,8 mg	2,67	12	15 6	2,35	0 06	1,25	19 66 mg = 36%

% = percentage of the injected dose.

is in contradiction with WYERS and VAN MUNSTER (1961) GARROW and WATERLOW (1959) and SCHWARTZKOPF and OEFF (1960) who stated the curve had an exponential course Further consideration of the curves described by WYERS and VAN MUNSTER (1961) and those by GARROW and WATERLOW (1959) who followed the disappearance of Evans blue over 8–35 days only is likely to reveal that when continued over a longer term those curves will not present an exponential course either CONOLLY and WOOD (1954) too, point out that the course of the disappearance curve is not an exponential one.

We demonstrated in dogs that after intravenous injection of Evans blue the dye is accumulated in several organs. Therefore we think it very likely that in man dye from depots may be restored to the circulation, which fact explains that the Evans blue disappearance curve has no exponential course.

As mentioned by WOLF (1952) and by MILLER and MILLER (1953) azo dyes have carcinogenic properties. For Evans blue this might be explained by the above-mentioned accumulation in certain organs. WILSON (1957) has shown that this dye has also a teratogenic effect on the foetus. For this reason some reserve in the use of T 1824 for diagnostic purposes would seem to be desirable

In three dogs (Table 3) we determined the quantity of T 18–4 in liver kidney spleen as well as that excreted in the bile 12 resp 19 days after intravenous injection. In total only 36–47% of the injected dose was recovered If in these dogs the quantity not recovered should be distributed diffusely over the remaining part of the body the various organs would present a dye concentration of circa 0.0015 mg/g tissue. Although accurate determination of lower concentrations is not possible qualitative demonstration is fairly easy In the lungs, heart, skeletal muscles and subcutaneous tissue hardly any dye was traced, the concentration of the dye being certainly far below 0 0015 mg/g tissue.

Hence we may conclude that Evans blue is not only excreted in the bile and accumulated in various organs, but also that a large amount must be broken down in the body

SUMMARY

The azo dye Evans blue (T 1824), although specifically and firmly bound to albumin, is no real tracer for this serum protein; the dye will disappear

by excretion we wondered whether T 1824 might be accumulated somewhere in the body

Now it is known that after intravenous injection of Evans blue the lymph nodes turn manifestly blue (LE VEEN and FISHMAN 1947) MILLER (1947) TOBIN and MOORE (1943) GARROW and WATERLOW (1959) LAWSON *et al* (1947) and CRUICKSHANK and WHITFIELD (1945) all assumed the reticuloendothelial system (RES) to be capable of retaining Evans blue according to ALLEN and ORAHOVATS (1950) and FREINKEL *et al* (1953) the dibasic amino-acids of the nucleoproteins are alleged to bind the dye CRUICKSHANK and WHITFIELD (1945) contended that the binding by the RES sets in very soon after injection (cat effect) GREGERSEN (1951) and others have disproved this assertion MOORE *et al* (1943) experimented with radio-active di bromo Evans blue in mice. 24 hours after injection they found a high level of radioactivity in the liver as well as in the intestinal mucosa and the faeces. With the techniques as described we did find in dogs high concentrations of Evans blue in the liver spleen kidneys and lymph nodes, especially in the mesenteric ones (Table 2) However only traces of dye were found to be present in the intestinal mucosa. This discrepancy with the findings of MOORE *et al.* (1943) could be explained by the fact that these authors measured radioactivity Presumably they determined mainly degradation products of the dye

In several dogs the Evans blue concentration appeared to be fairly constant in the organs when the investigation was made 5–20 days after intravenous injection of dye This was also the case when a gallbladder fistula was made or when the biliary duct was obstructed When however this investigation took place 32, respectively 38 days after injection (Table 2 dogs nr 12 and 16) lower concentrations were found in all organs.

From these experiments we may conclude that the bond between dye and albumin is severed after which the dye is stored in the organs, whereas the uncoloured albumin set free continues to take its normal part in metabolism In an investigation of 8 healthy men extended over a period of 65 days, we found that the plasma disappearance curve of Evans blue has certainly not an exponential type in decline but that in a logarithmical plot a parabola will be significantly more satisfying than a straight line (Fig 1) This

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from the serum more rapidly than ¹³¹J albumin does. Moreover our experiments showed that when the disappearance curves of normal adults are followed over a period of 60–65 days, they will not present an exponential course contrary to what is reported in the literature.

When investigating these phenomena in dogs we found that the dye was excreted in the bile and also to a small degree, in the intestines. However the quantities recovered from bile and faeces were too slight to account for the rapid disappearance of the dye.

Also we demonstrated accumulation of the dye in the liver spleen, kidneys, and lymph nodes. We think it very likely that from these depots dye is restored to the circulation thus preventing the serum disappearance curve to assume an *exponential course*. We determined in three dogs the amount of dye recovered in the body 1–19 days after intravenous injection.

From these findings the conclusion would seem justified that part of the dye is broken down in the body. As it is shown that azo dyes have carcinogenic and teratogenic properties and as we demonstrated that accumulation of T 18 4 occurs in various organs, some reserve in the use of this dye for diagnostic purposes would seem to be desirable.

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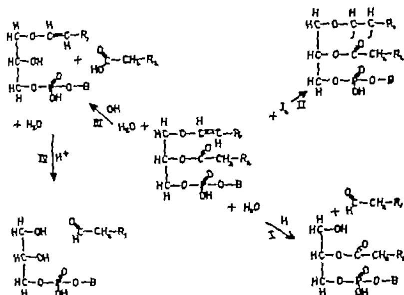


Fig. 1.

Diagrammatic representation of the conversion of plasmalogens by treatment of phospholipids according to the various methods for the determination of plasmalogens. For explanation see text.

phenylhydrazones formed are extracted with hexane. An aliquot is concentrated and diluted again with ethanol. The extinction of this solution is measured at 395 nm.

Results with this method are usually 10–15 pCt. too low but with a recent modification, proposed by PRIES and BÖTTCHER (1965) a 100 pCt. recovery of standard substances is reported.

After the definite proof of the α – β-unsaturated ether structure of native plasmalogens by RAPPORT and coworkers (RAPPORT *et al.* 1957; RAPPORT and FRANZL, 1957) Norton used the method of SIGGIA and EDSBERG (1948) for the determination of vinyl ethers by specific addition of I_2 (reaction II). This method has the advantage of being absolutely specific for plasmalogens under the conditions used. It has however not much been used, perhaps because of its relatively low sensitivity.

The methods described thus far do not discriminate between choline, ethanolamine and serine plasmalogens. For anyone

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METHOD FOR THE SIMULTANEOUS DETERMINATION OF PHOSPHOLIPIDS AND THEIR PLASMALOGENS THE ROLE OF PLASMALOGENS IN THE EMBRYONIC DEVELOPMENT OF THE BOVINE BRAIN

BY

R. E. DE ROOIJ AND G J M HOOGHWINKEL

1 INTRODUCTION

Since plasmalogens were discovered in 1924 by FEULGEN and VOIT (1924) various methods for their determination have been proposed (FEULGEN and GRÜNBERG 1939 WITTENBERG KOREY and SWENSON 1956 NORTON 1960 DAWSON 1960) The principles on which these methods are based are summarized in Fig 1

In the oldest and still widely used method of FEULGEN (1939 1951) (reaction I) plasmalogens are hydrolysed with 2 N HCl. The fatty aldehyde thus formed reacts, after neutralisation with NaOH with the fuchsine-sulphurous acid reagent of Schiff This method is very sensitive but it has several disadvantages. The reaction between the aldehyde and leucofuchsine has to take place in water and is dependent on the dispersion of the aldehyde Further difficulties arise with the extraction of the coloured compound prior to the colorimetric determination e g the formation of troublesome emulsions. As the reagent also interacts with short chain aldehydes, formed by oxydation of unsaturated fatty acids the values obtained by this method tend to be somewhat too high Several modifications (ANCHEL and WAELSCH 1944 EHRLICH *et al* 1948 GRAY and MACFARLANE 1958) have been described but none of these seems to be quite satisfactory

The technique devised by WITTENBERG KOREY and SWENSON uses the same principle. Hydrolysis is achieved with *0 1 N sulphuric* acid and followed by reaction with *p* nitrophenylhydrazine in ethanol at 70° for 20 min After addition of water the *p*-nitro-

breaks down lecithin and sphingomyelin fractions which are free from plasmalogens. Although we are not yet able to say how $HgCl_2$ reacts with phospholipids we think it justified to warn against its use in phospholipid research.

In 1959 MARINETTI, ERBLAND and STOTZ described a plasmalogen determination based on a paperchromatographic separation of the degradation products of phospholipid fractions after treatment with 90 pCt acetic acid, with or without addition of $HgCl_2$. They carried out phosphorus determinations in the chromatographic spots. Instead of measuring the aldehyde formed during acid hydrolysis, as in the methods of Feulgen and Wittenberg *et al.*, the amount of lysophospholipid produced by breakdown of the plasmalogens is determined. The method which we introduce can be seen as a modification of the Marinetti-method. It has the advantage of being much simpler thanks to the use of the tricomplex staining procedure which allows a direct determination of the plasmalogen content of a phospholipid mixture

2. MATERIALS AND METHODS

Human blood samples from healthy males and females were obtained by venous puncture In all cases clotting was prevented by addition of heparin (1 mg for 25 ml blood)

Red cells were separated by centrifuging for 20 min at 2000 rev/min. Plasma was removed with a Pasteur pipette and separately extracted. Erythrocytes were washed three times with 0 9 pCt. saline After washing the red cells were centrifuged sharply and the lipids were extracted from the cells with methanol, methanol chloroform (1 1 v/v), and chloroform. Each of these solvents was used twice in succession in liberal amounts (about 50 ml each time for 10 ml plasma or erythrocytes). The extracts were combined and dried *in vacuo* at a temperature not exceeding 35° The residue was dissolved in a few ml of chloroform methanol (4 : 1 v/v).

Fresh ox heart obtained from the slaughterhouse was cut into pieces of about 1 cm³ These pieces were homogenized with methanol (100 ml for 15 g of tissue). The mixture was kept at room temperature for 20 min. The solvent was decanted through a sintered glass filter and the residue was extracted with the same volume of methanol-chloroform (1 1 v/v). After 20 min this

interested in the distribution of plasmalogens in the various phospholipid fractions this is a serious handicap. Several authors (GRAY and MACFARLANE l. c. WEBSTER, 1960. THIELE et al. 1960), have determined plasmalogen concentrations in phospholipid fractions after chromatographic separation. But according to the rather labile nature of the vinyl etheric bond such procedures are liable to give erroneous results (PRIES and VAN GENT unpublished results). Moreover they require as a rule much material and are thus unsuitable for routine analysis.

In 1960 Dawson devised a method for the complete evaluation of the composition of a complex phospholipid mixture. The sample to be analysed is first hydrolysed with alkali (reaction III). Fatty acid residues are split off and diacyl phospholipids are converted to water soluble glycerophosphate esters while plasmalogens form lysoplasmalogens. After separation the enol ethers of the lysoplasmalogens are hydrolysed under acid conditions and again glycero phosphate esters are formed (reaction IV). Remaining sphingomyelins and other phospholipids can be hydrolysed with concentrated HCl. The water soluble glycero-phosphate esters are separated with paper chromatography and determined by measuring the amount of phosphorus in each spot. In a later paper DAWSON et al. (1962) describe several modifications including the use of $HgCl_2$ in reaction IV necessary to split cyclic acetals eventually formed from the lysoplasmalogens.

Although this method has the advantage of specifying the different forms in which plasmalogens can occur it is rather laborious and requires a fairly large amount of material (16 μ moles).

The method of selective hydrolysis has been criticized by PIETRUSZKO and GRAY (1962) who found a small loss of aldehyde during alkaline hydrolysis. The same paper describes the formation of a cyclic product during acid hydrolysis of lysophospholipid which is much more resistant to acid hydrolysis than the expected lyso product. Moreover the rate of hydrolysis of acyl groups is not the same for di acyl phospholipids and plasmalogens (RENKONEN 1963).

DAVENPORT and DAWSON (1962) have found that formation of the cyclic acetal can be greatly reduced though not entirely prevented by addition of $HgCl_2$ to the trichloroacetic acid used for hydrolysis. We have found however that $HgCl_2$ in acetic acid

previous day The hydrolysed extract is applied half as many times as the original extract. Chromatograms are run as described by HOOGHWINKEL and VAN NIEKERK (1960b) during 6 to 8 hours. They are dried in a stream of air and stained in a bath containing 0 005 pCt. Ponceau red (Edical Supra Ponceau 4 RS, ICI) 0.. pCt. $UO_2(NO_3)_2$ and 0 01 N HCl, during 10 to 16 h. In this bath only amphoteric phospholipids are stained. After drying between filter paper the spots are outlined with a pencil and cut out very carefully with a pair of scissors. Each spot is placed in a 25 ml erlenmeyer flask and the dyestuff is extracted with 3 ml 50 pCt tert. butanol containing 0.6 N HCl. After a few hours the extinction of the solution is determined at 510 nm against the solvent as a blank. Background colour which is very slight, does not have to be determined because the presence of lipid in a spot completely prevents the weak interaction of the staining reagent with the fibers in the paper

Usually four components are present, viz. ethanolamine cephalin, lecithin, sphingomyelin and lysolecithin. In the hydrolysed material the cephalin and/or lecithin spots are diminished and the sphingomyelin and lysolecithin spots are increased in comparison with the original sample (Fig 2) The increase of the sphingomyelin spot is due to the presence of lysocephalin which has approximately the same Rf value as sphingomyelin.

As the reaction between phospholipid uranyl nitrate and Ponceau red is stoechiometric (HOOGHWINKEL and VAN NIEKERK, 1960a) the amount of dyestuff bound is proportional to the molar amount of phospholipid involved. Therefore the extinction values measured correspond with the molecular distribution of the phospholipids.

From these extinction values the percentage composition before and after hydrolysis is calculated. The plasmalogen content is found from the differences in composition between hydrolysed and non-hydrolysed material.

3. RESULTS

Data on phospholipid composition of different tissues are compiled in Table 1 Ethanolamine plasmalogen concentrations are calculated from either the decrease of cephalin or the increase of sphingomyelin and the choline plasmalogen concentrations are calculated from the decrease of lecithin or increase of lysolecithin.

procedure was repeated and a final extraction with the same volume of chloroform followed. The combined extracts were dried and the residue was dissolved in chloroform methanol (4 : 1 v/v).

Brain phospholipids were extracted by blending the sample with chloroform methanol (2 : 1 v/v) using 20 ml solvent for 1 g tissue. The homogenate was kept at room temperature during 1 to 1½ hours. Insoluble material was removed by filtration; the extract was dried and the residue dissolved in chloroform methanol (2 : 1 v/v).

All lipid extracts were stored at −20° and remained essentially unchanged for several weeks.

Lipid phosphorus was determined with the modified method of Zinzadse according to HOOGHWINKEL and VAN NIEKERK (1960c).

Paper chromatography was carried out on silica impregnated paper (S & S 2043b) using as solvent di-isobutylketone-acetic acid-water (40 : 25 : 5 v/v/v) (HOOGHWINKEL *et al.* 1959).

For impregnation the procedure of MARINETTI and STOTZ (1956) was slightly modified: 310 g silica gel (Mallinckrodt Chemical Co.) was carefully dissolved in 1 liter ~2 N NaOH; the solution was made up to 1.7 l with water. Strips of S & S filter paper (12 × 24 cm) were impregnated for 5 min in this solution, blotted between filter paper and immersed in 6 N HCl for 5 min. The strips were then transferred to a second bath of 6 N HCl where they remained for 25 min. The strips were thoroughly washed with tap water and finally rinsed with distilled water, dried in the air and stored in a desiccator.

2.1. PLASMALOGEN DETERMINATION

An aliquot (usually 0.2 ml) of the lipid sample containing about 2 μ moles of phospholipid is dried in a stream of nitrogen. The residue is dissolved in 0.1 ml 90 pCt. acetic acid. In this medium a specific hydrolysis of plasmalogens to aldehydes and lysophospholipids takes place (GRAY 1958, 1960). The acetic acid solution is placed in a bath of 37° and kept there overnight. For the chromatographic separation two impregnated filter papers are used. On both papers four spots are applied using the original extract and the acetic acid solution in alternation. The sample is usually applied 6–10 times. The number of applications is derived from a spot test according to HOOGHWINKEL *et al.* (l.c.) made the

TABLE I

Phospholipid composition of some tissues.

Tissue		Percentage composition of phospholipid before (b) and after (a) hydrolysis				Amount of plasmalogen as percentage of fraction indicated			
		C	L	S	LL	C *incr*	C *decr*	L *incr*	L *decr*
Human erythrocytes	b.	25.5	40.5	30.5	3.2	34.5	32.8	1.7	2.8
	a.	17.4	39.3	39.4	3.9				
Human plasma	b.	trace	53.6	25.8	20.6			3.7	3.5
	a.	trace	51.7	25.7	22.6				
Human white matter	b.	37.7	39.4	22.9	trace	77.2	77.5		
	a.	8.6	29.4	62.0	trace				
Human grey matter	b.	33.6	52.1	14.3	trace	35.7	37.5		
	a.	21.1	52.6	26.3	trace				
Bovine brain	b.	40.5	40.1	18.2	1.1	50.5	50.6	4.0	6.2
	a.	17.5	37.6	42.3	2.7				
Bovine corpus callosum	b.	38.4	34.3	27.3		69.0	60.7		
	a.	12.8	33.4	53.8					
Bovine heart muscle	b.	34.0	54.3	9.0	2.7	50.7	50.9	49.0	49.0
	a.	16.7	27.7	26.2	29.3				

Abbreviations See explanation Fig. 2. The numbers under the heading *incr* (increase) are calculated from the increase of the lysoproduct, those under the heading *decr* (decrease) are calculated from the decrease of the parent compounds.

They comprise 2 pCt or less of the lecithin fraction which means less than 1 pCt of the total phospholipids. From table * it is also evident that a steady increase of the sphingomyelin occurs from the 8th month on This means that plasmalogen increase preceeds sphingomyelin increase during embryonic development How these observations are related to the process of myelination will be dealt with in a further study

Striking differences are also observed by comparing the plasmalogen content of bovine heart muscle at various ages. Analyses were made in three cases, one of embryonic heart muscle one of calf heart and one of adult ox heart. Results of these analyses are shown in Table 3.

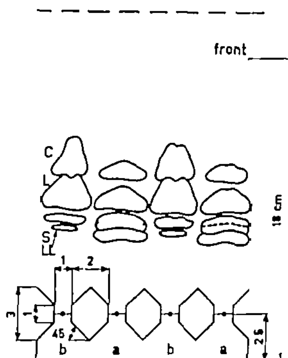


Fig. *

Chromatogram of phospholipids of bovine heart muscle before and after hydrolysis. C, L, S and LL denote ethanolamine cephalin, lecithin, sphingomyelin and lysolecithin respectively. This picture also gives the dimension of the chromatographic paper used for the separation. In the hydrolysed phospholipids the sphingomyelin spot is divided into two regions, the upper one representing sphingomyelin, the lower one representing lyso-ethanolamine cephalin.

Table 2 shows the changes in plasmalogen content of bovine brain during embryonic development. The age of the embryo is expressed *in months after conception*. The age was determined from Fig. 3. In this figure the estimated age of the embryo is plotted against total wet weight of brain. The estimation was done by officials of the slaughterhouse on general appearance of the foetus. A steady increase of the amount of ethanolamine plasmalogen *is observed up to the 8th month*, after that period no significant further increase can be demonstrated. Lecithin plasmalogens are found only in very small amounts. Calculation from either the decrease of lecithin or the increase of lysolecithin results in values which are not sufficiently reliable to include them in the table.

choline plasmalogens. In the first place we would mention the simplicity of the procedure which is demonstrated by the chromatographic separation of the products on filter paper without any further treatment of the lipids after the aldehyde groups are split off from their plasmalogens. Secondly we would draw attention

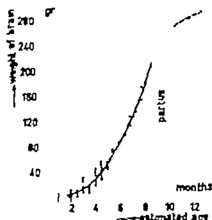


Fig. 2.

The curve gives the relation between the total weight of the brain and the estimated age of the fetuses.

TABLE 3

Phospholipid composition of bovine heart muscle.

	Percentage composition of phospholipids before (b) and after (a) hydrolysis				Amount of plasmalogen as percentage of fraction indicated		
	C	L	S	LL	C	L	T
Embryo	b. 34.3	51.1	12.2	2.8	61	13	27
	a. 14.1	42.9	33.7	8.2			
Calf	b. 34.3	55.1	8.7	1.9	49	20	38
	a. 17.3	34.0	25.0	21.7			
Ox	b. 34.0	54.3	9.0	2.7	61	49	45
	a. 16.7	27.7	20.2	20.3			

Abbreviations: See Fig. 2. T denotes total.

TABLE –
Phospholipid composition of developing bovine brain.

Age in months			Percentage composition of phospholipids before (b) and after (a) hydrolysis: C	L	S	LL	Amount of ethanolamine plasmalogen as percentage of fraction indicated.*: O	T
1½	(4)*	b	33 0	58.7	4 8	2.0	35	1
		a.	21 0	58.3	16.3	3.8		
2½	(10)	b	33.0	59 8	4 8	–.4	37	1
		a.	21 4	57 8	17 5	3 3		
4	(30)	b	31 6	63.4	3.3	1 9	38	12
		a.	*0 1	01 3	15.8	2.8		
5½	(74)	b.	34.3	59 7	4 0	–.0	43	16
		a	19 0	58 9	18 5	3 0		
6½	(98)	b.	36.4	50.0	5 6	1.5	49	18
		a.	19 1	55 0	23 4	6		
7	(115)	b	34 8	57.3	6.3	1 0	51	13
		a.	17 7	54 9	*4 6	–.8		
7½	(134)	b	31 7	00 8	5 7	1 8	54	17
		a.	15 3	58 5	3 7	* 0		
8	(153)	b	34.3	58.1	7 6	trace	54	18
		a.	10 4	57 0	*0 0	trace		
9	(*18)	b	35 7	51 9	1–.3	trace	5*	18
		a.	17 8	50 7	31.5	trace		
3 (after birth)	(342)	b	40.–	45.2	14 6	trace	54	22
		a.	18.3	45 3	30 4	trace		
adult	(418)	b.	40.5	40 1	18.2	1 1	58	4
		a.	17.5	37 6	4–.3	2 7		

See Fig * T denotes total.
Numbers in parentheses give total wet weight of brain used
* The values given are the mean of the values obtained by using both methods of calculation (see Table 1 and the text)

Although changes in phospholipid composition are but small considerable changes in the plasmalogen amount – increase – and their distribution – decrease of ethanolamine plasmalogen and increase of lecithin plasmalogen – occur during the process of ageing

4 DISCUSSION

The method presented in this paper has some advantages over other methods in use for the determination of ethanolamine and

choline plasmalogens. In the first place we would mention the simplicity of the procedure which is demonstrated by the chromatographic separation of the products on filter paper without any further treatment of the lipids after the aldehyde groups are split off from their plasmalogens. Secondly we would draw attention

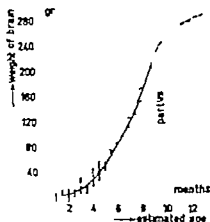


Fig. 2.

The curve gives the relation between the total weight of the brain and the estimated age of the fetuses.

TABLE 3

Phospholipid composition of bovine heart muscle.

	Percentage composition of phospholipids before (b) and after ( ) hydrolysis				Amount of plasmalogen as percentage of fraction indicated		
	C	L	S	LL	C	L	T
Embryo	b. 34.2	51.1	12.2	2.5	61	13	27
	a. 14.1	43.9	33.7	8.3			
Calf	b. 34.3	55.1	8.7	1.9	48	30	35
	a. 17.3	34.0	25.0	23.7			
Ox	b. 34.0	54.3	9.0	2.7	51	40	45
	a. 16.7	27.7	26.3	29.3			

Abbreviations See Fig 2. T denotes total.

to the fact that using this procedure the ethanolamine and choline phospholipid distribution in mol percentages and the plasmalogen content of these various phospholipids are obtained at the same time. As a disadvantage we can mention the fact that only those phospholipids and phospholipid plasmalogens which are stainable on chromatograms with the tricomplex staining procedure are assayed. However, as it is known that in most tissues serine phospholipids are present in relatively low concentrations compared with choline and ethanolamine phospholipids, we think that this method will be valuable for the study of various biological problems of lipid metabolism.

Concerning the sensitivity of the method we may say that the values for the plasmalogen content of the phospholipids obtained by this method are of course determined within the limits of the paperchromatographic quantitation method. By our experience with the quantitation of phospholipids with this method we could evaluate that the phospholipid distribution measured is accurate to within a few per Cent. This was studied for red cell and plasma lipids of various animal species (HOOGWINKEL *et al.* 1960; HOOGHWINKEL and BRUYN 1966; HOOGHWINKEL, DE ROOIJ and DANKMEIJER, 1965).

The plasmalogen percentages of the various phospholipids, however, are calculated by subtraction of two percentage distribution values obtained after paper chromatographic separation. The methodological error of the quantitation method thus occurs twice in the plasmalogen values. This disadvantage is overcome rather well by using both calculating procedures (see Table 2) and taking for the final plasmalogen percentage the mean value of both approaches. Plasmalogen percentages below 3 pCt are not to be regarded as very reliable and only give a reasonable approximation.

In our examples which are included to demonstrate the usefulness of the method we clearly demonstrate that the method can be a very good aid to the study of biological problems. We can see that in the embryonic development of bovine brain, the observed increase of plasmalogen percentage of the ethanolamine phospholipid precedes the increase of sphingomyelin. Sphingomyelin itself more or less starts to increase when histologically myelin can be detected in the tissue.

By studying various bovine heart lipid samples we found that the phospholipid distribution was the same in heart samples of animals of various ages. The plasmalogen percentages of the ethanolamine and choline phospholipids however undergo significant changes during the process of ageing.

Both examples indicate the importance of plasmalogens in the lipid structures in biological development of both organs. We think this is of general importance in studying the physico-chemical properties of biological membrane structures as such and also of changes in these properties during development and in pathology.

ACKNOWLEDGEMENT

This study was carried out with support of the Netherlands Organisation for the advancement of Pure Research (Z.W.O.) and the Foundation for Chemical Research in the Netherlands (S.O.N.).

SUMMARY

1. A survey is given of the (different) methods described in the literature with a discussion of their advantages and limitations.
2. A new method is introduced in which plasmalogens are determined by calculating the differences between the composition of the phospholipid mixture before and after hydrolysis with 90 pCt. acetic acid.
   This composition is determined by a quantitative paper chromatographic technique using the tricomplex staining.
3. Data on the changes in phospholipid composition during myelination of bovine brain and in the process of ageing of bovine heart, obtained with this method, are given.

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TIME-RESPONSE EXPERIMENTS WITH ANTICOAGULANTS ON RATS

BY

P. M. L. TAMMES *), P. E. LOOSJES **) AND R. WIJNEN *)

INTRODUCTION

In the last fifteen years anticoagulants have become the preferred chemical agents for rodent control. Their chief advantage is that they do not introduce a repellent property in baits. They may and should be used for several days in succession until the animals are killed as a result of internal bleeding. The dose, though repeated, is low and thus limits the hazards for men and domestic animals. Incidental intakes are usually without lethal effect. Moreover from the point of view of visual symptoms of intoxication killing rats by anticoagulants is less repulsive than e.g. by Scilla (LOOSJES, 1959).

The only drawback of their application is the long time that elapses before a desirable effect is obtained. In general the treatment must continue until no more bait is eaten, which may take two weeks of administration or even more, especially when black (ship) rats (*Rattus rattus L.*) or house mice (*Mus musculus L.*) are concerned. For testing these rodenticides it is necessary to apply them during a certain period, and to incorporate the time factor in the assessment. Methods of testing anticoagulants as rodenticides were described, e.g. by HAYS and GAINES (1950), by BENTLEY (1955) and by LOOSJES (1959).

A study was carried out on the possibilities of arriving at an exact interpretation of data from experiments concerning the relationship between time and effect.

USING TIME AS A MEASURE OF TOXICITY

BLISS (1940) mentions that "a time factor may be involved not only as a stimulus, such that the length of exposure to a given

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concentration determines the amount fixed within the organism, but also as the measured response to a given dose of poison which may or may not involve a time factor in its administration

Experiments on chronic poisoning involve three variables the dose or concentration of the poison the exposure period, and the effect observed (mortality) To simplify the comparison of various poisons or animal species in this three-dimensional relation one may choose a fixed level of one variable and study the relation of the others by means of two-dimensional graphs. We may thus have

a the dosage/mortality graph based on a fixed exposure period
b the time/mortality graph based on a fixed dose (administered either at the beginning of the experiment or in the form of daily doses)
c the time/dose graph based on a constant effect, e.g a 50% mortality

The considerations below mainly refer to b and c, the time factor being the variable measured The following model might then apply

Suppose that there is no elimination of the poison absorbed and that death occurs when a certain amount has accumulated, this amount being a random variable Choosing a mortality of 50% as the level of effect the expectation would be that the product of d (daily dose) and t (number of days) would be constant i.e. $d \times t = c$. On a linear scale this relation would be represented by the positive branch of an orthogonal hyperbola. Taking logarithms the equation is $\log d + \log t = \log c$, so that on a logarithmic scale the graph would be a straight line meeting the coordinate axes at an angle of 45°

This can be explained as follows. In Fig 1 assuming angles of 45 $p+q$ will always equal AB or AC On two-sided log paper $\log p + \log q$ will equal $\log AB$ or $\log AC$ However as $\log p + \log q = \log p \times q$ the value of $p \times q$ will be constant for the line BC If p is the concentration in the food or the daily dose and q is the response time and if there is a constant value of the product $p \times q$ then the line will approach both axes at angles of 45°

Deviation of the 45° line to a hyperbole with one or both axis will indicate an elimination of the poison or of the effect at lower doses or an approach to a limit of minimum response time at higher doses.

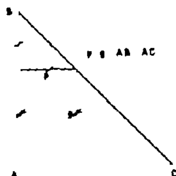


Fig. 1 Explanation in text.

In pharmacology the method of plotting dosage-time responses on two-sided logarithmic paper is used when dealing with various dosage levels. This is probably done because it was found that in many cases straight lines were obtained. Two examples may be taken from pharmacology

In toxicity experiments with liver flukes VAN NOORDWIJK *et al.* (1963) obtained a straight line in a concentration time graph plotted on two-sided log paper the time being probably too short for a measurable elimination. In experiments with a carcinogenous substance fed to rats during their entire life DRUCKREY and co-workers (1962) also obtained a straight line of 45° in a log time-log dosage graph indicating that there is a summation of effect without apparent elimination, a summation of hits" or a situation of irreversible damage. The same method is used for the measurement of toxicity for fishes in polluted water and examples were described by JONES (1964).

For calculation and plotting graphs it is more convenient to use median values rather than mean values. A median value can be estimated when for instance 50% of the population is still alive and this often allows terminating the experiments long before all animals of a batch are killed. A median value can also directly be used for log or non-log plotting In case of a mean value it will make a difference whether it is a log or a non-log average of several log values.

Another method of plotting was used by POWERS (1917) in experiments with several toxic substances on the goldfish. He plotted the reciprocal values of the lethal time, 100/(lethal time in days) which he called velocity of fatality.

For calculation this method is very convenient because very long survival times are reduced to near zero and thus enables the calculation of a mean with reciprocals of infinite values. There is however no reason to exclude the higher values and the method, though first used was later abandoned by the authors.

MATERIAL AND METHODS

Experiments were carried out with brown rats *(Rattus norvegicus Berkenh.)* and the white laboratory variety and with black ship rats *(Rattus rattus L.)* Each batch consisted of 5 animals, which were given a fixed concentration of the anticoagulant in the laboratory food. The lethal exposure time for each animal was recorded and the median lethal time of each batch assessed. *Experiments were terminated after 40 days, natural mortality being negligible during this period.* One experiment was carried out with 10 animals per batch and continued until the last animal died after 210 days.

RESULTS

The table below represents the results of the experiment with 10 animals per batch continued until the last animal died.

TABLE 1

ppm in food	survival time in days per animal										median survival time
3.2	7	7	8.	8.	8.	9	11	11	13	14	8
[illegible]4	5	9	13	14	15.	19	[illegible]1	[illegible]9.	31	41	15
1.6	13.	13	13	[illegible]	[illegible]3	36.	45	147	[illegible]08	210	23

Attention should be drawn to the larger variation at lower dosages and the long survival times for the last three animals. Natural mortality can be ignored as it starts after this period. The three animals mentioned also died of internal bleeding. Results

are plotted in Fig. 2 in a dosage-time graph. A tabulation of results of various other experiments is given in Table 2 which also demonstrates that some series are killed at lower concentrations than others.

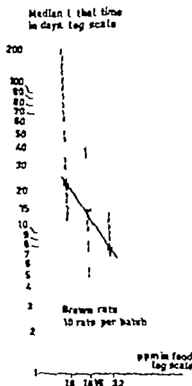


Fig. 2. Dosage-time graph (× = median lethal time, and the dotted lines present the variation in lethal times).

STATISTICAL SIGNIFICANCE OF RESULTS

From Table 2 it is clear that at high concentrations of poison and short survival times the differences between batches are small, probably due to a minimum lethal time which is needed for these poisons. At very low concentrations there are clear differences between poisons, e.g. when comparing the 3.1 ppm level of Endrocid with the other compounds. In those cases in which the survival

TABLE 2

Explanation in text

Lethal time in days after regular feeding with anticoagulants

Compound	Warfarin (standard)		Endrocid	Compound O	Compound E.	Compound B	Endrocid
Species →	Black rat ♂♀	Brown rat ♀♀	Brown rat ○♀	Brown rat ♀♀	Brown Rat ♀♀	Brown Rat ♀♀	White rat ○○
ppm in food ↓							
	6	4	4	4	7	10	5
	8	5	6	5	8	10	6
400	0	6	0	8	9	11	6
	10	6	7	8	0	13	7
	11	10	8	8	11	19	8
	5	7	4	5	5	6	4
00	5	7	5	6	8	0	6
	8	8	5	7	9	10	7
	9	10	6	7	9	11	8
	13	11	7	7	13	11	8
	7	0	6	0	15	7	6
	9	8	6	7	15	10	6
100	10	0	7	8	36	12	6
	14	9	0	0	37	15	8
	14	9	19	12	30	18	8
	0	6	5	6	0	1	6
	9	7	0	8	37	*8	7
50	12	7	6	9	38	*9	7
	14	9	6	13	>40	>40	8
	10	10	8	14	>40	~40	9
	10	6	4	6		19	5
	11	9	5	10	all	1	6
5	3	0	5	13	>40	38	7
	24	9	6	13		>40	7
	>40	10	8	10		>40	8
	15	7	5	8		10	6
	>40	9	6	12		>40	6
12.5	>40	10	7	10		>40	7
	>40	10	7	>40		>40	7
	>40	11	9	>40		>40	8

TABLE 2 continued

Compound	Warfarin (standard)		Endrocid	Compound O	Compound E	Compound B	Endrocid
Species →	Black rat ♂♀	Brown rat ♀♀	Brown rat ♂♀	Brown rat ♀♀	Brown Rat ♀♀	Brown Rat ♀♀	White rat ♂♂
ppm in food ↓	21	7	3			6	7
	>40	8	5	all		>40	7
6.3	>40	16	5	>40		>40	9
	>40	23	8			>40	9
	>40	>40	9			>40	10
		14	7				8
	all	24	8			all	9
3.1	>40	>40	8			>40	10
		>40	10				11
		>40	12				13
			12				22
		all	16				36
1.6		>40	26				>40
			>40				>40
			>40				>40
			all				all
0.8			>40				>40

times show an overlap the significance of the results should be assessed statistically The test chosen is Wilcoxon s two-sample test. In Table 3 rankings of survival times are given for some comparisons. There is some evidence that experiments with 5 animals are insufficiently indicative and that it would probably be better to increase the number of animals from 5 to 8.

In order to illustrate such a test a comparison is given between Endrocid (e) and Warfarin (w) for 4 levels of toxicant in the food, with the figures taken from Table 2. W is the test statistic (rank sum). Statistical evidence is accepted from $P < 0.05$.

It follows that the no-difference (zero) hypothesis can be rejected for the levels of 25 12.5 and 3 1 ppm but not for the 6.3 ppm level.

A larger number of animals might reveal whether at this latter concentration the difference is also significant.

The results of the experiments tabulated in Table 2 are represented as graphs in Figs 3, 4 and 5.

TABLE 3

Two-sample test of Wilcoxon for survival times of Endrocid (e) & Warfarin (w) on brown rats

ppm in food	ranking from long to short lethal time	W (rank sum)	P value
25	w w w w o o o o o	16.5	<0.02 sign.
	(w)		
12.5	w w w w o o	17.5	<0.05 sign.
	w o o		
	o		
6.3	w w w o o w w	21	>0.3 not sign.
	o o		
	o		
3.1	w w w w w o o o o o	15	<0.01 sign.

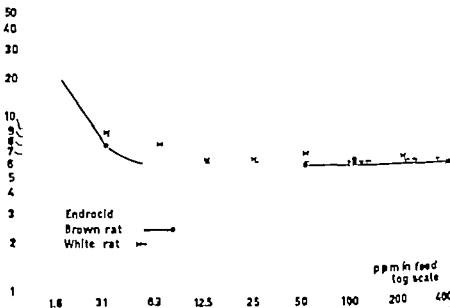


Fig 3 The plotted lines show that for Endrocid there is only a small if any difference between the brown rat and its white laboratory variety

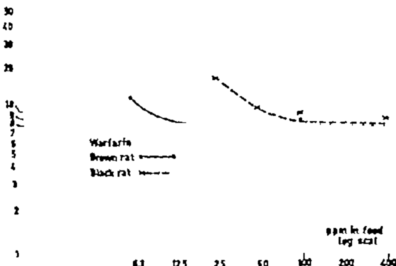


Fig. 4. For Warfarin there is significant difference between the brown rat and the black rat at low levels of poisoning. This method can also be used to detect resistant strains in rats.

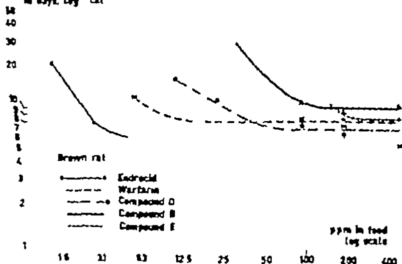


Fig. 6. Difference in effect of various compounds. Some differences are significant, others are not (when the lines are too close).

INTERPRETATION OF RESULTS

Using time as a criterion it was possible to obtain information on various problems regarding the evaluation of various anticoagulants on two species of rats. The differences are not due to the amount of the active chemical in the compound as there is only limited variation in this respect.

In the graphs the horizontal lines indicate that at higher levels of poisoning a limit, the minimum lethal time, is approached. This limit appears to vary between compounds.

The region where differences can be detected most clearly is the upward slope at the lower levels of poisoning. Statistical treatment is possible e.g. at the same level of poisoning in using the distribution free two-sample test of Wilcoxon. For small differences the size of the batches must be increased for obtaining a significant difference.

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SUMMARY

Various dosage levels of anticoagulants were continuously fed to white laboratory, brown and black (ship)rats. Using time as a criterion, differences were found and could be tested statistically by a distribution free method. Results were plotted on a log time-log concentration graph, using median lethal time as a criterion. Significant differences were observed between some of the compounds when tested on the brown rat. A significant difference in sensitivity between the brown rat and the black rat was demonstrated. The method seems also suitable to detect resistant strains in rat populations.

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Department of Dermatology of the Roman Catholic University Nijmegen

THE INTERACTION OF SOME METAL IONS WITH THE UTERUS OF THE GUINEA PIG

BY

D SPRUIT AND J P KUIPER

1 INTRODUCTION

The contraction of the guinea pig uterus is generally used in the technique of SCHULTZ and DALE (see e g JANSE, 1960) in order to investigate properties of materials which cause allergic reactions. This method could not be used in the investigation of nickel ions, as non-sensitized uteri contracted in their presence The nickel concentration was about 0 1 to 1 mmol Ni^{++}/l An even more peculiar feature was that the reactivity of the normal uterus to repeated doses of the Ni^{++} decreased even after rinsing with the complexing agent EDTA a decreasing reactivity would only be expected in the case of a hypersensitive uterus. In addition previous exposure to Ni^{++} does not influence the response of the uterus to other agonists, e.g histamine Co^{++} in contrast does not cause contraction of the uterus

These observations are interesting as it is already known that the nodal action potential is very much prolonged by the same Ni^{++}-concentrations and it has been concluded that this effect is due to delayed and reduced inactivation of sodium permeability and delayed increase of potassium permeability (MEVES 1963). Similar effects were observed by HASHIMURA and OSA (1963) using Co^{++}-solutions at concentrations ten times higher than the nickel concentrations Therefore Ni^{++} and Co^{++} were both investigated

2. METHODS

The uteri were cut out of the guinea pig suspended in the apparatus according to SCHULTZ and DALE (Fig 1) and rinsed

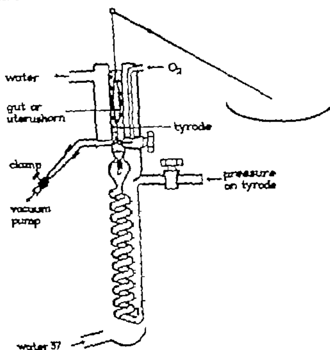


Fig. 1 Schultz Dale apparatus.

with Tyrode solution. The length of the uteri was about 2½ cm the diameter varied from 2 to 4 mm. 20 ml Tyrode solution surrounded the uterus at a temperature of 37 °C. The contraction of the uterus was registered. The Tyrode solution was stirred by bubbling air through it. As soon as the uterus relaxed the metal ion was added to the solution by pipetting a concentrated solution of the chloride.

At any desired moment e.g. 5 min later some liquid was sampled in order to determine the amount of metal ion present. The amount of nickel was determined by measuring the intensity of the colour developed by a dimethylglyoxim reagent at 445 mμ cobalt was determined by the method of WISE and BRANDT (1954) and uranyl according to FRANÇOIS (1958).

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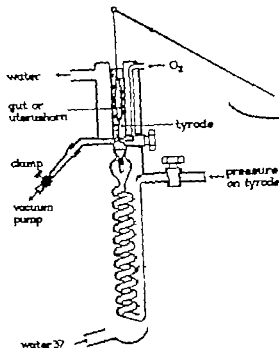


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At any desired moment, e.g. 5 min later some liquid was sampled in order to determine the amount of metal ion present. The amount of nickel was determined by measuring the intensity of the colour developed by a dimethylglyoxim reagent at 445 mμ cobalt was determined by the method of WISE and BRANDT (1954) and uranyl according to FRANÇOIS (1958)

The Tyrode solution was renewed by draining it into a container and pouring new Tyrode solution into the apparatus, repeating these manipulations three times This flushing process took about ten sec

At the end of each experiment the uterus was weighed. The weight of the uterine tubes filled with liquid, varied from 60 to 250 mg the air-dry material of the lightest ones being about 10 mg In order to determine the nickel content of the uterus after the experiment was finished (as a control) it was placed in 4 ml of a mixture of concentrated HCl and HNO_3 (1 1) carbonized dissolved in 4 ml HCl+HNO_3 and the nitric acid removed by evaporating with HCl The residue was dissolved in a little HCl and the nickel determined colorimetrically using dimethylglyoxim. The nickel content of the uteri always correlated with the calculated content (Figs. 4 5 and 6)

3 RESULTS

3 1 THE UPTAKE OF Ni^{++} $(UO_2)^{++}$ AND Co^{++} BY THE UTERUS

The rate of nickel uptake by the uterus from the surrounding

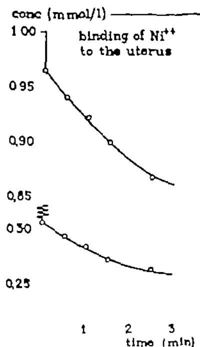


Fig 3 The uptake of Ni by the uterus.

Tyrode solution was measured by sampling some liquid every 30 sec after the nickel had been added to the solution. Fig 2 represents the decreasing Ni-concentration of the surrounding liquid. After about 3 min the Ni-concentration of the solution had become nearly constant no more nickel was absorbed by the uterus. As this period of about 3 min is very short it may be concluded that the metal ion had probably been absorbed on the outside of the uterus and had not yet penetrated into the cells (see VAN STEVENINCK, 1962)

Cobalt and uranyl behave similarly (Fig 3)

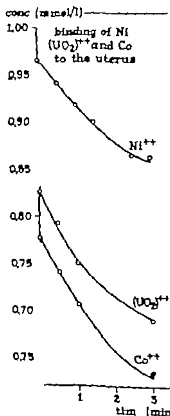


Fig. 3. The uptak of Ni^{++} $(UO_2)^{++}$ and Co^{++} by the uterus.

At the end of the 3 min exposure the average concentration of the metal ion in the (total, wet) uterus was calculated, and

compared with the metal ion concentration of the surrounding solution The metal ion was shown to have been concentrated about 30 times

The Ni-concentrations shown in Fig 2 produce a contraction of the uterus as do the uranyl concentrations shown in Fig 3. The cobalt however does not cause a contraction of the uterus. The contraction of the uterus by nickel was at its maximum when the amount of nickel added increased the concentration of Ni in the surrounding liquid to about 1 mmol/l Yet the uterus was still able to contract as it could be stimulated by histamine. A small contraction of the uterus was produced when the concentration of nickel in the surrounding liquid was about 0 3 mmol Ni/l. A second weak contraction could sometimes be observed with this low concentration when the experiment was repeated after changing the Tyrode solution but this occurred only shortly after the first contraction

At the critical concentration producing maximum contraction (1 mmol Ni/l) and at the higher concentration of 10 mmol Ni/l the uptake of nickel by the uterus was about the same The uterus was left in the solution for exactly 3 min The Tyrode solution was then changed three times and the uterus left in this solution for ten min After this time 0 07 mmol Ni/l was found in the solution whether the original contraction had been caused by a concentration of 1 mmol Ni/l or by 10 mmol Ni/l. When the initial concentration was 0 3 mmol Ni/l however only 0 02 mmol Ni/l was found in the elution liquid. The maximum contraction of the uterus by Ni^{++} seems to be correlated with this maximum adsorption.

3 2 THE RELEASE OF METAL ION FROM THE UTERUS

The elution of nickel from the uterus was studied at pH 7.5 and pH 5 The uterus was suspended in the Tyrode solution for exactly 5 min after the nickel ion had been added. During this period a steady state was established as was shown before. The surrounding solution was then quickly replaced by fresh Tyrode solution (without Ni) Ten minutes later a steady state was again obtained and the solution was replaced once more by a fresh one. These rinsings were repeated several times. Meanwhile a few ml of solution were removed in order to determine the nickel concentrations (see Fig 4)

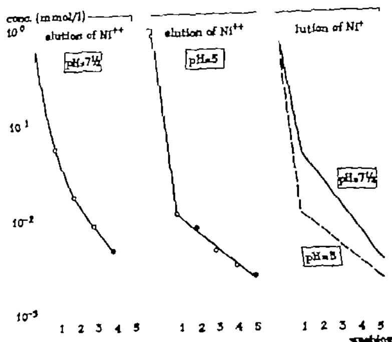


Fig. 4. The elution of Ni^{++} from the uterus.

At the lower pH less nickel was adsorbed by the uterus, as is evident from the nickel concentrations of the first washing. At pH 7.5 it was about 0 07 mmol at pH 5 only 0 01 mmol Ni/l (Fig 4) Yet at pH 5 the elution of nickel from the uterus by successive rinsings was not proceeding as easily as at pH 7 5 The subsequent Tyrode solutions contain quite appreciable amounts of nickel, showing that it was being released quite slowly The effect is demonstrated clearly in the third graph of Fig 4.

The amount of nickel absorbed may be increased when the incubation time of the 1 mmol Ni/l at the beginning is extended from 5 to 10 min (Fig 5) The last traces of nickel are removed from the uterus only with difficulty Consequently much more nickel penetrates into the organ when the incubation time is lengthened. The penetration apparently proceeds more slowly than does the adsorption to the organ.

Fig. 6 shows the influence of the cobalt ion on the adsorption of nickel by the uterus. The cobalt is concentrated by the uterus,

compared with the metal ion concentration of the surrounding solution The metal ion was shown to have been concentrated about 30 times.

The Ni-concentrations shown in Fig 2 produce a contraction of the uterus as do the uranyl concentrations shown in Fig 3 The cobalt however does not cause a contraction of the uterus. The contraction of the uterus by nickel was at its maximum, when the amount of nickel added increased the concentration of Ni in the surrounding liquid to about 1 mmol/l. Yet the uterus was still able to contract as it could be stimulated by histamine. A small contraction of the uterus was produced when the concentration of nickel in the surrounding liquid was about 0.3 mmol Ni/l. A second weak contraction could sometimes be observed with this low concentration when the experiment was repeated after changing the Tyrode solution but this occurred only shortly after the first contraction

At the critical concentration producing maximum contraction (1 mmol Ni/l) and at the higher concentration of 10 mmol Ni/l the uptake of nickel by the uterus was about the same The uterus was left in the solution for exactly 3 min The Tyrode solution was then changed three times and the uterus left in this solution for ten min After this time 0 07 mmol Ni/l was found in the solution whether the original contraction had been caused by a concentration of 1 mmol Ni/l or by 10 mmol Ni/l When the initial concentration was 0 3 mmol Ni/l however only 0 02 mmol Ni/l was found in the elution liquid The maximum contraction of the uterus by Ni^{++} seems to be correlated with this maximum adsorption.

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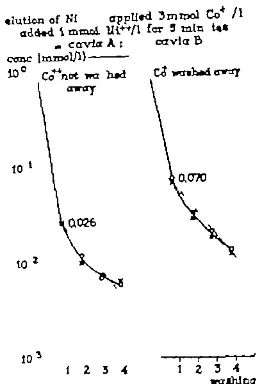


Fig. 6. The influence of Co^{++} on the adsorption of Ni^{++}

proceeded with the same speed whether cobalt was present or not, as is obvious from the equal slopes of both graphs (broken lines) of Fig. 6

SUMMARY

1. The addition of Ni^{++} or $(UO_2)^{++}$ up to a concentration of 0.1 mmol to 1 mmol/l causes a contraction of the uterus. Co^{++} does not contract the uterus, even at higher concentrations. The contraction produced by Ni cannot be repeated, unless very low concentrations are repeated within short time

2. Maximum contraction and maximum adsorption both occur at about 1 mmol N^{++}/l.

3. The presence of Co^{++} in the uterus decreases the contraction produced by nickel to some extent and at the same time less nickel is adsorbed. Previous contact of the uterus with cobalt does not prevent contraction by nickel, after the cobalt has been rinsed from the uterus.

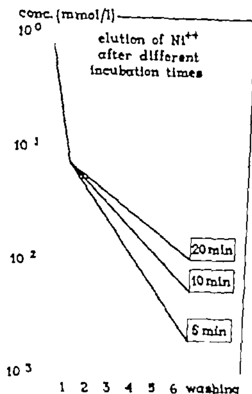


Fig 5. The elution of Ni + from the uterus, as influenced by various incubation times with Ni

as was mentioned before although it is not able to cause a contraction It can be expected that cobalt and nickel ions may be exchangeable (VAN STEVENINCK 1962) When a concentration of 3 mmol Co/l was rinsed from the uterus before 1 mmol Ni/l was applied the uterus behaved quite normally the ability of the nickel ions to cause a contraction was not diminished and nickel reacted in the same way as was described before (Fig 6 second graph) Yet less nickel was adsorbed when the nickel was added to the solution after the cobalt has been present for 5 min and was not eluted. A weak contraction of the uterus occurred in these circumstances. The cobalt concentration had not completely blocked the adsorption of nickel but had lessened it to about one third as might be expected when the two ions competed with each other about equally (Fig 6 first graph) Apparently the adsorbed concentration of nickel ions caused the contraction of the uterus The elution of the nickel ion from the organ into the solution

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Departments of Dermatology of the Universities of Utrecht (RHC) and Nijmegen (DS and JPK)

STIMULATION OF ENZYME ACTIVITY IN THE UTERUS OF THE GUINEA PIG BY NICKEL IONS

BY

R. H. CORMANE[1]), D. SPRUIT AND J. P. KUIPER

1. INTRODUCTION

The observation that nickel ions cause a contraction of the uterus of guinea pigs (SPRUIT and KUIPER, 1967) led to an investigation into the influence of these ions on histochemically demonstrable enzymes in that organ.

Nickel usually inhibits the activity of enzymes, if any effect can be observed (VALLEE, 1961). We found, however, a characteristic stimulation of the activity of alkaline phosphatase in some circumstances. In the human uterus this enzyme has been observed to occur with variable activity and to be especially localized in the muscle fibres (OBER, 1950). It has been suggested, that the enzyme plays a role in glycogenesis and in the transport of organic substances across cell membranes (HOYACK VAN PAPENDRECHT 1963, HERTIG *et al.* 1958).

2. EXPERIMENTAL

The guinea pig uteri were suspended in the apparatus according to SCHULTZ and DALE, as described before (SPRUIT and KUIPER, 1967). Some contractions were produced by the addition of histamine to the surrounding Tyrode solution. The histamine was rinsed from the uterus with Tyrode solution. A calculated amount of nickel or cobalt chloride was then added to the Tyrode solution to obtain the final concentrations shown in Table 1. When the uterus had contracted, it was allowed to relax before it was

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4. The metal ion is adsorbed by the uterus within 3 min, but it penetrates much more slowly into the interior.

5. Only minor amounts of nickel are adsorbed at pH 5, compared with pH 7.5.

6. A uterus which has been stimulated by nickel, can not be regenerated, even by the addition of the complexing agent EDTA.

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Not only was the activity of the alkaline phosphatase of the myometrium of uteri *b* and *f* different from normal but the activity and localization in the endometrium had also changed. In normal sections alkaline phosphatase activity was observed in the interstitium of the uteri only and no activity was observed inside cells of the endometrium or at cell membranes (Fig 1 *a*). Even the uteri, treated with 3 mmol Co/l, showed no appreciable alkaline phosphatase activity in the myometrium and the endometrium nor was any activity of the enzyme observed at cell membranes. At the cell membranes of the glandular epithelium of uteri *b* and *f* however intense activity of alkaline phosphatase was seen (Fig 1 *f*). The interior of the cells again showed no activity

3 DISCUSSION

Because the activity of alkaline phosphatase was determined by the method of GOMORI (1952) and since during this treatment cobalt sulphide is being precipitated, the presence of nickel or cobalt originating from the experiments might possibly give rise to blackening not originating from alkaline phosphatase activity However the concentrations of nickel and cobalt used in these experiments were low Thus, a concentration of 0 2 mmol Ni/l did not blacken the sections. A concentration of 3 mmol Co/l may have blackened the sections to such an extent that the activity observed was somewhat increased" (Table 1) Therefore the nickel originating from the 3 mmol Ni/l solution cannot have blackened the sections appreciably Besides, the rinsed uteri *f* were blackened as much as uteri *b*. Consequently the really "much increased" activity of the alkaline phosphatase observed in *b* and *f* must have originated from the enzyme itself.

If the concentration of nickel ions was in excess of that required for maximum contraction and maximum adsorption, the activity of the alkaline phosphatase in the interstitium of myo- and endometrium was very much increased. It is striking, moreover that a very strong enzyme activity was found at the cell membranes in the endometrium, where it was not present before The presence of nickel changed the alkaline phosphatase activity very much, but only when it was present in excess.

Incubation with fresh Tyrode solution for half an hour

transferred to a tube and immersed in liquid nitrogen for quick freezing. Because the lowest nickel concentrations and all cobalt concentrations did not produce a contraction of the uterus (Table 1 *d* and *e*) a period of 5 min was allowed to pass before these uteri were taken from the Tyrode solution to be frozen. During this 5 minute period the metal ion will have been adsorbed to the uterus.

In Table 1 uteri *f* were not treated in the same way as uteri *b*

TABLE 1

Contraction and alkaline phosphatase activity of the uterus of the guinea pig with some metal ion concentrations.

	uterus stimulated by Ni +	Co++	contraction	alkaline phosphatase activity of the myometrium
a	—	—	—	normal
b	3 mmol/l	—	+	much increased
c	0.3 mmol/l	—	+	normal
d	0.01 mmol/l	—	—	normal
e	—	3 mmol/l	—	somewhat increased
f	3 mmol/l and rinsed by Tyrode solution	—	+	much increased

and the other uteri as they were very well rinsed with Tyrode solution to remove all nickel from the uteri. In view of the results of previous experiments (SPRUIT and KUIPER, 1967) this was achieved by washing three times immediately after the contraction with an interval of 2 min between washes. this procedure was repeated for half an hour.

The uteri were investigated histochemically for some hydrolyzing enzymes, alkaline and acid phosphatases AMP-ase ADP-ase, ATP ase and polyphosphatase as in earlier investigations of human skin by CORMANE and KALSBEEK (1963). Only the alkaline phosphatase activity of some uteri appeared to be different from the activity of the normal histamine-stimulated uteri. In this study the method of GOMORI (1952) was applied. The results of the estimations are recorded in Table 1.

(experiments *f*) did not restore the low intensity of the alkaline phosphatase activity nor remove it from the cell membranes. As a consequence the change in alkaline phosphatase activity by the transient presence of nickel is not a temporary one. This lasting enzyme activity correlates with the non-restorable contractility of the uterus after treatment with such nickel concentrations and might perhaps be correlated with variations in membrane permeability (see MEYER, 1963; HERTIG *et al.* 1958).

SUMMARY

The alkaline phosphatase activity of the interstitium of myo- and endometrium is much increased when the uterus of the guinea pig has been exposed for a few minutes to a nickel concentration exceeding the concentration required for maximum contraction and maximum adsorption (> 1 mmol Ni^{++}/l). In addition enzyme activity is found at the cell membranes of the glandular epithelium in the endometrium where it was not found before.

The alkaline phosphatase activity of uteri, exposed to Co^{++} does not deviate from normal.

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Fig. 1 Sections of the uterus of the guinea pig. Cells coloured by hematoxylin-eosin; alkaline phosphatase activity traced by CoS (Gomori-method).

a) uterus stimulated by histamine only; no nickel present. No alkaline phosphatase activity can be observed at cell membranes of the glandular epithelium in the endometrium (arrow).

b) uterus stimulated by 3 mmol Ni/l; immediately after the contraction the Ni was rinsed completely for ½ h. Phosphatase activity is now observed at cell membranes. For explanation see text.





f

EXPERIMENTAL DESIGN

Three sows of the breed Dutch Land pig, were used for the experiment. They were 3 to 4 years of age and farrowed at the same time. The estimated weights varied from 200–250 kg The data on the sows and piglets are summarised in Table 1.

TABLE 1

sow nr	date of birth sow	calculated date of birth of piglets	actual dat of birth of piglets	number of piglets alive	dead	total
I	15-11 1963	7-4-1966	11-4-1966	7	—	7
II	12-4-1963	8-4-1966	12-4 1966	16	2	18
III	10-12-1962	3-4-1966	5-4-1966	15	4	19

On 14th March 1966 the sows were put together in a sty and washed with Soabexan. Sows I and II were chosen as experimental animals, sow III was the control animal. A relatively pure preparation of aflatoxin-B_1 with a concentration of 10 μg B_1 per g was supplied by the R.I.V In practice, the amount of aflatoxin-B_1 found in pig rations was never more than 200 μg per kg and therefore this concentration was chosen for the rations of the experimental animals. It had been determined beforehand that the pig food concentrate to be used in the experiment was free from aflatoxin. The composition of the rations in percentages was 25 maize 10 barley 5 milo 7,5 oats, 10 peas, 5 linseed meal, 5 soybean-oil meal solvent, 5 corngluten feed, 10 wheat middlings, 10 alfalfa, 5 fishmeal (67% crude protein), 0,25 dicalciumphosphate, 0 75 vitamin B preparation, 0 15 vitamin AD_3, 1 5 mineral mixture.

The sows were fed at 8.30 a.m. and at 5.00 p.m. 2 kg food was given at each feed, an amount which they consumed easily The aflatoxin-B_1 concentrate was pre-mixed with a small part of the weighed meal in the trough. Only after the sows had consumed this quantity completely was the remainder of the meal given, mixed up with the necessary water This ensured that the experimental sows consumed 200 μg aflatoxin B_1 per kg food. Drinking-water was given ad libitum

The experimental period started on March 14th at the afternoon feeding

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Stichting Instituut voor Moderne Veevoeding „De Schothorst" Hoogland (U.) The Netherlands

CHANGES IN THE FATTY ACID PATTERN OF THE LIVER OF YOUNG PIGLETS UNDER THE INFLUENCE OF AFLATOXIN B_1

BY

DRS. W. A. G. VEEN

in co-operation with the Rijksinstituut voor de Volksgezondheid (R.I.V.) at Bilthoven and the Stichting voor Onderzoek van Pluimvee en Varkens (Pluva) at Amersfoort, The Netherlands

In the past years much literature has appeared on the toxic influence of the mycotoxins (aflatoxin) produced by Aspergillus flavus. The presence of aflatoxin can often be demonstrated in groundnuts. VAN KOETSVELD[1]) (personal communication) has shown its occurrence in some cases in pig concentrates in the Netherlands, but never at a concentration greater than about 200 μg aflatoxin B_1 per kg food. One of the many symptoms resulting from feeding with aflatoxin is fatty degeneration of the liver, as has been shown by many investigators, more recently by BUTLER and WIGGLESWORTH (1966) in rats. FRIEDMAN (1964) reviewed the literature on aflatoxin intoxications. Amongst the most sensitive animals are turkeys, ducks, pigs, calves and rats in which among others damage of liver parenchymal cells, proliferation of bile-ducts and hepatoma can occur. LOOSMORE and HARDING (1961) described liver damage in pigs after feeding Brazilian groundnuts.

This report deals with the influence of a daily intake of 800 μg aflatoxin B_1 by pregnant and lactating sows on the health of the piglets. The influence of an extra supply of 100 μg aflatoxin B_1 to sucking piglets was also studied.

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1) Present address: Stichting Pluva, Amersfoort.

and DYER (1959). For the preparation of the methyl esters of fatty acids about 150 mg fat was saponified with 4 ml 0.5 n methanol/NaOH, after which it was boiled with 5 ml BF_3-methanol ($\pm$ 14% BF_3)[1] for 2 min. After addition of 10 ml saturated NaCl-solution the esters were extracted with 3 portions of about 4 ml petroleum-ether (b.p. 40–60 °C). The combined extracts were filtered through waterproof filter paper and evaporated to dryness in a rotating evaporator (temperature of the water bath not over 50 °C). Up to this point the method of METCALFE, SCHMITZ and PELKA (1966) was followed. The mixture of methyl esters was now purified by thin layer chromatography. The mixture – dissolved in 0.3 to 0.4 ml petroleum-ether – was put on 1 mm thick plates of silica-gel-G (Merck).

After developing the chromatogram in petroleum-ether/diethyl ether/acetic acid 85 : 15 : 1 the spots of the methyl esters were marked under UV-light and then scraped off the plate and eluted with pentane[2]. The methyl-ester solutions were analysed with a Carlo Erba gas chromatograph, model D, with a flame ionisation detector. The column was made of glass, 1.60 m long and filled with 19.5% PEGA[3] on a solid support of Chromosorb-W 80–100 mesh[3]. The temperature of the column was 197 °C and the flow of the carrier gas, nitrogen, was 55 ml per minute.

The relative quantities of the fatty acids were determined by calculating the products of retention time and peak height. For the identification according to the method of WOODFORD and VAN GENT (1960) some standard fatty acids[4] were used. The list of relative retention times, given by FARQUHAR *et al.* (1959) and the technique of micro-hydrogenation also described by FARQUHAR *et al.* were used.

RESULTS

HISTOLOGICAL EXAMINATION

The results of the histological examination of the livers by Dr. E. J. Voûte of the Pluva are summarised in Table 3.

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[1]) Boron trifluoride methanol complex (BDH).
[2]) Pentan 99% ("für die Chromatographie") E. Merck A.G.
[3]) Carlo Erba.
[4]) E. Merck A.G.

Directly after birth the piglets were allowed to drink colostrum. In this period 3 piglets died one from litter I on April 1?th and two from litter II one on 14th and one on 15th April Pathological anatomical examinations were carried out The experimental sows consumed no concentrate during the first few days after farrowing Later on normal feeding was resumed On April 19th the piglets in litters I and II were divided into two equivalent groups according to weight In this period the active immunisation might be expected to have started so that they were then most sensitive to infection and intoxication The classification of the piglets is given in Table –

TABLE 2

Sow	sow milk only			+ aflatoxin B_1		
nr	nr	sex	weight (g)	nr	sex	weight (g)
I	1	sow	3 50		boar	3840
	4	boar	3530	3	sow	3330
	6	sow	3 00			
	10	sow	1940	5	boar	805
	1?	boar	390	7	sow	5 0
	13	sow	000	8	boar	15?0
II	14	sow	030	9	boar	470
	15	sow	1500	11	sow	400
	18	boar	0?0	16	boar	1930
				17	sow	1090

In the afternoon of April 19th the piglets of the experimental group received aflatoxin B_1 for the first time Every piglet swallowed a capsule containing 50 μg aflatoxin B_1 twice a day From April 19th piglets from the two experimental groups and the control group (litter III) were killed and examined histologically and pathologically The organs and carcasses were examined for traces of aflatoxin B_1 and its metabolites. The sow milk was also examined. The *fatty acid pattern of heart and liver fat* was examined in the laboratory of De Schothorst

ANALYTICAL METHODS

The fat from the hearts and livers of the piglets was extracted with chloroform and methanol according to the method of BLIGH

and DYER (1959) For the preparation of the methyl esters of fatty acids about 150 mg fat was saponified with 4 ml 0 5 n methanol/NaOH, after which it was boiled with 5 ml BF_3-methanol (± 14% BF_3)[1]) for 2 min. After addition of 10 ml saturated NaCl-solution the esters were extracted with 3 portions of about 8 ml petroleum-ether (b p 40–60 °C). The combined extracts were filtered through waterproof filter-paper and evaporated to dryness in a rotating evaporator (temperature of the water bath not over 60 °C). Up to this point the method of METCALFE, SCHMITZ and PELKA (1966) was followed. The mixture of methyl esters was now purified by thin layer chromatography The mixture – dissolved in 0,3 to 0 4 ml petroleum-ether – was put on 1 mm thick plates of silica-gel-G (Merck)

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	4	boar	3530	3	sow	3330
	6	sow	3 60			
	10	sow	1940	5	boar	805
	12	boar	380	7	sow	‿570
	13	sow	660	8	boar	15°0
II	14	sow	°050	9	boar	470
	15	sow	1500	11	sow	400
	18	boar	0°0	16	boar	1980
				17	sow	1690

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ANALYTICAL METHODS

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acid and of the remaining acids with 20 and 22 C-atoms and two or more double bonds decreased. From three weeks after birth the fatty acid pattern appeared to remain quite constant in all piglets. Any influence of intake of aflatoxin-B_1 was not observed. Some differences in composition existed between the litters (Table 4).

TABLE 4

Average percentages of the most important fatty acids in the heart from 3 weeks after birth. (In brackets the number of piglets)

	litter III (3)	litter I (afla toxin) (2)	litter I (without) (3)	litter I (total)	litter II (afla toxin) (3)	litter II (without) (2)	litter II (total)
Palmitic acid	21 1	19,2	18,5	18,8	19 4	18,7	19 0
Palmitoleic acid	6,2	5,8	6,9	6,4	7,2	7 0	7 1
Stearic acid	12,7	11,1	11,5	11,3	10,7	9,7	10,2
Oleic acid	31 1	31,0	29,8	31 1	28,1	26,7	27 4
Linoleic acid	14,3	13,9	16,5	15,4	12 7	14 1	13,4
Linolenic acid	1,5	1 7	2,2	2,0	1 4	1,6	1,5
Arachidonic acid	4,0	5,5	4,8	5,1	2,7	3 7	3,2
Remaining unsaturated C20 and C22 fatty acids	2,9	3,9	3,7	3 8	2,4	2,9	2,6

LIVER

The fatty acid pattern of the liver also changed during the first weeks after birth, but it must be noted, that the analyses were performed on livers of piglets which had died. Histological examination of the piglets in litter II that died after 2 days, showed a fatty infiltration. After about 3 weeks the picture remained quite constant for those piglets that had not received extra aflatoxin, although fluctuations in litters I and II were somewhat greater than in litter III The changes shortly after birth concerned linoleic and stearic acid which increased, in contrast to oleic and palmitoleic acid which decreased. On the whole there appeared to be differences between the three litters similar to those found in the fatty acid pattern of the heart. The changes in percentages of palmitic, stearic

TABLE 3

Sow nr	number of piglets examined	age of piglets (days)	number of days with aflatoxin B_1	histological observations in the liver: + extra aflatoxin B_1	histological observations in the liver: without extra aflatoxin B^a-B_1
I	1	1	0		normal
II	1	1	0		some disorganisation
	1	2	0		fatty infiltration
I	1	8	0		degeneration
II	1	6	0		disorganisation + some centres of degeneration
III	1	14	0		normal
I	2	23	15	tendency to fatty degeneration	normal
II	2	21	15	disorganisation + connective tissue + beginning of bile-duct proliferation	normal
III	1	29	0		some disorganisation
I	2	37	29	degeneration + bile-duct proliferation	excess formation of connective tissue
II	2	35	29	degeneration	normal
III	1	43	0		normal
I	1	51	43		normal
II	2	49	43	beginning of degeneration	normal some enlarged bile-ducts
III	1	57	0		normal

CHEMICAL EXAMINATION

In the following account the fatty acids are expressed as relative quantities in the fatty acid mixture

HEART

In the first weeks of life the fatty acid pattern changed quite considerably. The percentages of palmitic, palmitoleic, oleic and linolenic acid increased; the percentages of stearic and arachidonic

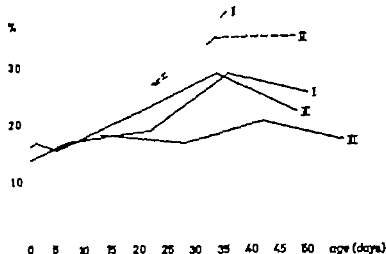


Fig. 2.
The percentage of stearic acid in the fatty acid mixture from the liver at different ages.
----- + aflatoxin-B_1

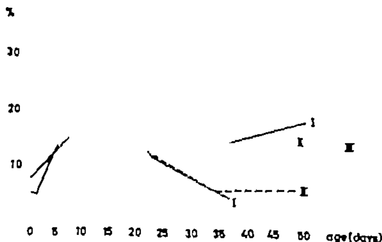


Fig. 3.
The percentage of linoleic acid in the fatty acid mixture from the liver at different ages.
--- } + aflatoxin-B_1

linoleic and arachidonic acid with age have been reproduced in graphs (Figs 1 to 4) On the basis of these graphs it seems justifiable

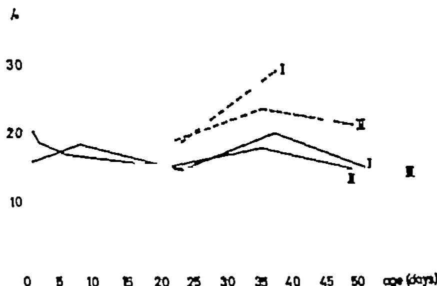


Fig 1
The percentage of palmitic acid in the fatty acid mixture from the liver at different ages.
– – – – – + aflatoxin B_1

to take the means of the values from an age of 3 weeks. But it is clear that the piglets receiving extra aflatoxin acquired a totally different fatty acid pattern This is even more evident in Fig 5 where the percentages of palmitic palmitoleic stearic, oleic linoleic linolenic and arachidonic acid and the remaining fatty acids with 20 and 22 C-atoms with two or more double bonds are reproduced in block graphs The piglets receiving aflatoxin B_1 during 0 *9 and 43 days respectively had a much higher percentage of palmitic and stearic acid The piglets in litter II also had a higher percentage of oleic acid Palmitoleic acid was somewhat increased in all cases at least as compared with the piglets in litter I and II that had not received aflatoxin B_1 The poly unsaturated acids were decreased

DISCUSSION

In the first weeks of life changes occurred in the fatty acid pattern of the heart and liver of piglets in litters I and II The fatty acid

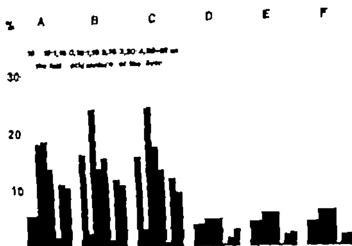


Fig 5

The percentages of palmitic, palmitoleic, stearic, oleic, linoleic, linolenic and arachidonic acid and the fatty acids with 20 and 22 C-atoms and two or more double bonds.

A Average values for 3 piglets from litter III (age 29 43, 57 days).
B Average values for 3 piglets from litter I (age 23, 37 51 days), without aflatoxin B_1.
C Average values for 3 piglets from litter II (age 21 35, 59 days) without aflatoxin-B
D Values for one piglet from litter I (age 37 days) after 29 days of aflatoxin B_1
E Values for one piglet from letter II (age 35 days) after 29 days of aflatoxin-B_1
F Values for one piglet from litter II (age 49 days), after 43 days of aflatoxin-B_1.

pattern of the liver did not diverge much from those of piglets in litter III that were killed later on (Figs. 1 to 4) The piglet in litter I that died after 1 day showed a strikingly low percentage of linoleic acid (Fig 3). This could be connected with a difference in fatty acid composition between very young and adult tissues. The low percentage of linoleic acid was also found in the piglets in litter II that died after 1 and 2 days but here histological abnormalities were also found, disorganisation and fatty infiltration respectively

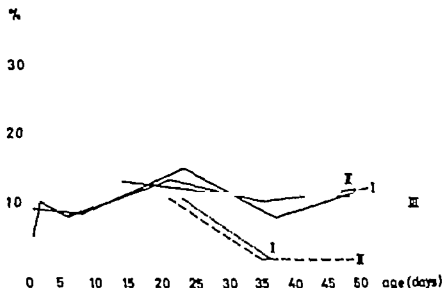


Fig. 4

The percentage of arachidonic acid in the fatty acid mixture from the liver at different ages.

- - - - - } + aflatoxin B_1
- - - - }

pattern in the heart of one piglet in the control litter (III) that was killed at 14 days of age diverged in some degree from those of other piglets in this litter killed later on. But it was comparable with the patterns of piglets in litter I and II that died at 8 and 6 days of age respectively. This suggests that these are normal changes in the first weeks of life which are not related to the administration of aflatoxin B_1 to the sows. These changes are not surprising because it is probable that the placenta of sows is only moderately permeable for free fatty acids (LEAT 1966) so that the fatty acid composition of foetal tissues can diverge markedly from that of adult tissues. After birth the fatty acid composition of the colostrum starts to play a role in that of the tissues of the piglets.

As regards the liver the results of the analyses concerning the first weeks of life are more difficult to interpret. Of the piglets examined before April 10th only the livers of one piglet in litter I (died after 1 day) and of one piglet in litter III (killed after 14 days) were histologically completely normal. Of the latter the fatty acid

which eat no more than 800 µg aflatoxin B_1 per day in the ration. For young piglets a daily intake of 100 µg aflatoxin B_1 over a longer period of time may upset the fat metabolism.

SUMMARY

A daily supply of 100 µg aflatoxin B to suckling piglets led t changes in the fatty acid pattern of the liver The percentages of saturated and mono-unsaturated fatty acids increased and the percentages of poly unsaturated fatty acids decreased with time. Histologically degeneration of the liver was observed. Some piglets, born from sows, that received 800 µg aflatoxin-B_1 p.d. during the last three weeks of pregnancy showed degeneration of the liver shortly after birth. The sucking piglets kept alive for a longer time, showed no histological and chemical changes in the liver at different ages.

There was no influence of aflatoxin-B_1 on the fatty acid pattern of the heart.

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If we consider the results of the histological examination then it is clear that of the piglets in litter I and II that remained alive more than 14 days and received no extra aflatoxin B_1 the livers were normal and similar to those of the piglets in the control litter. The fatty acid pattern of all these piglets was rather similar except in regard to stearic acid (Fig. 2). One might conclude that aflatoxin B_1 fed to sows does not have any influence on the piglets, but the histological abnormalities of the liver found in the piglets, examined in the first weeks of life indicate that caution is necessary in drawing these conclusions (Table 3). It can be assumed that there was no intoxication via the sows milk.

Neither aflatoxin B_1 nor a metabolite of it could be shown in the sows milk, examined at the R.I.V. If we bear in mind the quantities of aflatoxin B_1 administered this could not be expected. VAN DER LINDE *et al.* (1964) used much larger quantities to find a metabolite of aflatoxin in the milk of cows.

The administration of extra aflatoxin B_1 to some piglets led to striking changes in the fatty acid pattern of the liver. With time the changes were more pronounced. Bearing in mind the *composition* of the liver fat of the piglets in litter II which had received aflatoxin B_1 during 29 and 43 days respectively there is an indication that the composition remains constant in the end. The sort of changes, an increase of the percentages of saturated and mono-unsaturated fatty acids and a decrease of the poly-unsaturated fatty acids points to a fatty infiltration (Fig. 5). But this *is not shown histologically in all the cases*. In *only* one of these piglets was a tendency to fatty degeneration found (litter I). There was no clear correlation between duration of intake of aflatoxin B_1 and the histological changes. It is suggested that the chemical changes found can be demonstrated earlier in the liver than histological changes. It is striking that ZWART and HARDING (1964) in an experiment with black pigs in Ghana observed a strong fatty infiltration in the liver in early weaned pigs after one feeding of 400–450 g of maize brans infected with Aspergillus flavus. How much aflatoxin these piglets consumed with this ration is not mentioned.

An explanation of our findings can not be given at this time.

As a tentative conclusion to our investigations it can be stated, that there is no danger of intoxication of piglets suckled by sows

ronidase activity during the healing process in wounded rats and animals treated with vitamin A and hydrocortisone.

MATERIAL AND METHODS

Albino rats (Carworth Farms) weighing an average of 200 g were divided into 4 treatment groups as follows 1) control, 2) vitamin A, 3) hydrocortisone and 4) vitamin A + hydrocortisone. In addition, fifteen, normal intact rats were included for evaluation of basal enzyme levels.

The hair was removed from the dorsal surface with an electric clippers and the skins were incised under light ether anesthesia. The midline incision was approximately 1½ long and located 2" below the interscapular region. The 50 mg vitamin A acetate and/or 5 mg hydrocortisone acetate (Merck, Sharp and Dome) were applied locally along the incision and the wounds were closed with stainless steel clips.

Five rats were killed by decapitation at 0 25 0.50 0 75 1 2, 4 7 10 14, 17 20 and 26 days. The wounds were rapidly removed by cutting 3 5 mm on either side of the incision (total width, 7 mm) A section of skin from the same dorsal area was taken from the normal intact, untreated animals. After weighing on an analytical balance a piece of the excised tissue (excluding the areas of the stainless steel wound clips) was quenched between two blocks of dry ice for 1 minute. The frozen skin was placed in a rubber finger cot and shattered by hitting it several times with a hammer The macerated tissue was transferred to a flask containing 10 ml of cold distilled water and homogenized with a Vir Tis No 45 homogenizer until a uniform suspension was obtained (approximately 5 min). The homogenate was diluted to a specific concentration (2%) and aliquots used in the β-glucuronidase procedure according to TALALAY FISHMAN and HUGGINS (1946). One unit of β-glucuronidase activity liberates 1 μg of phenolphthalein from phenolphthalein monoglucuronide in 1 hr at pH 4.5 and 38° C under standard conditions.

Dry weights were obtained by drying a piece of the wounded skin in a vacuum oven at 60° C for 24 hours.

The biochemical data was evaluated statistically by analysis of variance and the "F test for significance (SNEDECOR, 1956).

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THE INFLUENCE OF VITAMIN A AND/OR HYDROCORTISONE ON THE β-GLUCURONIDASE ACTIVITY OF HEALING WOUNDS IN RATS

BY

JOHN P MANNING AND GENE DIPASQUALE

INTRODUCTION

The histochemical characterization of β-glucuronidase activity in healing skin wounds has been investigated in the rat guinea pig iguana natterjack toad axolotl etc (RAEKALLIO 1963 1965 CABRINI and CARRANZA 1961 STOLK 1961 1962a b) Depending on the stage and degree of repair this lysosomal enzyme localizes primarily in fibroblasts leucocytes macrophages multinucleated giant cells and regenerating epidermis and can be detected as early as one hour after wound initiation In the regeneration process, the role of glucuronidase has been related to (1) an adaptive defense mechanism (catabolic activity in phagocytosis) (2) cellular proliferation and (3) formation of ground substance (RAEKALLIO 1963 1965 CABRINI and CARRANZA 1961 STOLK 1961 1962a, b). In this latter regard DUNPHY and UDUPA (1955) demonstrated that mucopolysaccharides which are necessary for deposition of adult collagen from the soluble nonfibrous protein material produced by connective tissue cells were significantly increased during the first 3 days of wound healing In addition PERUMAL, SAMY LAKSHMANAN JUNGALWALA and RAO (1960) WOLBACH (1947) WOLF and VARANDANI (1960) and WOLF (1962) have illustrated the importance of vitamin A in the synthesis of acid mucopolysaccharides Vitamin A is involved in the formation of an active sulfate (3-phosphoadenosyl-5-phosphosulfate) necessary for the sulfation of mucopolysaccharides (VARANDANI WOLF and JOHNSON 1960 SUNDARESAN and WOLF 1963)

The purpose of this investigation was to evaluate the β-glucu

rats receiving vitamin A + hydrocortisone the β-glucuronidase reaction peaked at day 10 and decreased during days 14–26 however the activity still remained significantly higher than the wounded controls (Fig 2).

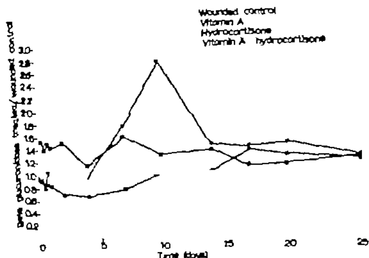


Fig. 2.

The ratio of the β-glucuronidase activity of vitamin A and/or hydrocortisone-treated animals versus the wounded controls Each point represents the average of five observations and the standard error of the mean did not vary more than ± 15 %.

The β-glucuronidase response to skin wounding is probably brought about by a number of contributing factors such as 1) catabolic hydrolytic activity of phagocytic elements 2) cellular hyperplasia and hypertrophy and 3) an anabolic role for the formation of ground substance (RAEKALLIO 1963 CABRINI and CARRANZA 1961 STOLK 1961 1962a, b) In this latter regard, DUNPHY and UDUPA (1955) suggested that wound healing could be divided in two parts (a) a productive phase (days 1–5) associated with maximum mucopolysaccharide synthesis, which is necessary for the deposition of adult collagen from a soluble, nonfibrous protein material produced by connective tissue cells (b) a collagen phase (day 5 to complete healing) representing the formation of the fibrotic elements of the wound matrix. Since β-glucuronidase peaks around the fourth day of the productive phase the

RESULTS AND DISCUSSION

This investigation demonstrated that biochemically determined β-glucuronidase which was increased by wounding per se peaked on the fourth day of healing (Fig 1) while the enzyme on days

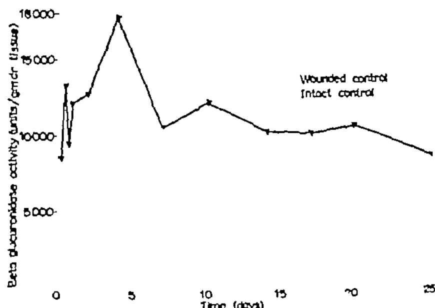


Fig 1

The β glucuronidase activity during the healing process in wounded control rats. Wounding per se significantly increased the enzyme level at all time periods. Each point represents the average of five observations and the standard error of the mean did not vary more than ± 15 %.

7–26 although still significantly higher than the unwounded controls stabilized at a lower level These results are similar to earlier histochemical reports on healing skin wounds of the rat, guinea pig iguana natterjack toad and axolotl (RAEKALLIO 1963 1965 CABRINI and CARRANZA 1961 STOLK, 1961 1962a b)

Compared to the wounded controls vitamin A maintained a significantly higher β-glucuronidase throughout the 26 day test period In hydrocortisone treated animals, however the enzyme activity was depressed during the first seven days and increased during days 17–26 of wound healing Such observations suggest that hydrocortisone may stabilize the subcellular structures by preventing production activation and/or release of the glucuronidase In contrast vitamin A would stimulate these processes In

SUMMARY

β-glucuronidase activity was evaluated in skin for 26 days during the healing process in wounded control rats and animals treated locally with vitamin A and/or hydrocortisone. Biochemically determined β-glucuronidase, which was increased by wounding per se peaked on the fourth day of healing, while the enzyme on days 7–26, although significantly higher than the unwounded controls, stabilized at a lower level. Compared to the wounded controls, vitamin A maintained a significantly higher β-glucuronidase level throughout the 26 day test period. In hydrocortisone-treated animals, however the enzyme level was depressed during the first seven days and increased during days 17 26 of wound healing. In rats receiving vitamin A + hydrocortisone, the β-glucuronidase reaction peaked at day 10 and decreased during days 14–26 however the activity was still greater than the wounded controls.

The results are discussed in relation to the role of β-glucuronidase in mucopolysaccharide synthesis and the formation of collagen.

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implication of this enzyme in mucopolysaccharide formation is *highly suggestive* In addition, there is an increased nucleic acid synthesis and glycogen deposition and these events are indicative of a hypermetabolic state (BRADFIELD 1951 JOHNSON and MCMINN 1960 TROTT and PEIKOFF 1963 WILLIAMSON and GUSCHLBAUER 1961 1965 GUSCHLBAUER and WILLIAMSON 1963)

The tensile strength was determined on 4 7 10 and 14 days after wound initiation in rats treated with vitamin A and/or hydrocortisone and the results were reported in an earlier communication (MANNING and DIPASQUALE 1967) In general wound strengths were similar in vitamin A and control animals. In contrast the locally applied glucocorticoid significantly lowered the tensile strength and this effect was only partially reversed by vitamin A If these results are compared to the enzymatic responses, no direct correlation can be made possibly because the events in healing are so complex and interrelated that the true connections with β glucuronidase are obscure For example the necessity of vitamin A for the synthesis of mucopolysaccharide has been previously demonstrated (PERUMAL *et al* 1960 WOLBACH 1914 WOLF and VARANDANI, 1960 WOLF 1962) thus one might expect that vitamin A administration to wounded rats would enhance mucopolysaccharide formation β glucuronidase activity and wound tensile strength In spite of the increased enzyme reaction however *the tensile strength over the 14 day period did not differ significantly* in the vitamin A treated and wound control rats. Apparently the sequence of events is so critically controlled (quantity and quality of substrates, time etc.) that the presence of any one component in excess amounts does not necessarily enhance wound healing On the other hand a deficiency in a decisive element (vitamin A, glycogen vitamin C RNA and DNA synthesis mucopolysaccharide formation) would most probably retard the healing process.

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The authors express their gratitude to Mrs Margaret C Butler Miss Shirley G Carter Mrs. Lenore V Tripp and Mrs. Lali Calenti for their technical assistance and Mr Neil Stasilli for the statistical analysis

In our search for the mechanism of the amino acid release from brain tissue by drugs we came across the non-specific alkaline phosphatase (E.C. 3.1.3.1) (DE WAART 1964).

Structural bound as it is (NOVIKOV 1964) we used this enzyme for our present experiments, measuring the activity by the hydrolysis of the substrate *p*-nitrophenylphosphate.

The data were recorded at physiological pH values, not only in order to simulate *in vivo* situations, but additionally the observed effects were actually greatest at pH 7.2–7.6. Thus in the course of our investigation on activity of *p*-nitrophenylphosphatase (pnpp) in homogenates and suspensions of subcellular particles we obtained evidence that phenothiazines affect the integrity of the membranes of brain tissue especially.

The results, which we have presented in preliminary communications (DE WAART *et al.*, 1966a, b), will be described in full in this paper.

METHODS AND MATERIALS[1])

Two months old male albino rats, which had free access to food and water, were killed by decapitation.

Cerebral hemispheres, liver and kidney were immediately removed and pooled homogenates of each organ were made in cold 0.25 M sucrose in a Potter Elvehjem homogenizer with teflon pestle. Final concentrations of homogenates were brain 10%, liver 5% and kidney 2.5% weight/volume. All subsequent operations were performed at temperatures of 0–4 C. Homogenates were either used directly or differentially centrifuged at 1000 × g and 8000 × g in Phywe Elspirouett and at 105 000 × g in Phywe ultracentrifuge P 30 K. The pellet from 10 min 1000 × g was washed once with 0.25 M sucrose and subjected to another 10 min at 1000 × g. This sediment was called nuclear fraction. From combined supernatants sedimented in 30 min at 8000 × g the crude mitochondrial fraction and subsequently in 60 min at 105 000 × g the microsomal fraction were obtained. The remaining supernatant

[1]) Phenothiazines were kindly provided by "Specia", Paris, and Orphenadrine by Brocades-Stheeman & Pharmacia" Haarlem. Other drugs and chemicals were commercially obtained.

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EFFECT OF SOME DRUGS ON p-NITROPHENYLPHOSPHATASE ACTIVITY IN BRAIN AND LIVER TISSUE OF THE RAT WITH SPECIAL REFERENCE TO INFLUENCE OF PHENOTHIAZINES ON MEMBRANES OF BRAIN TISSUE

BY

C DE WAART TH M BROGT AND J H SIETSMA

INTRODUCTION

From previous investigations in this laboratory DE WAART (1958a) reported that in rat brain slices histamine could antagonize the inhibition of oxygen uptake caused by several dimethylamine ethyl benzhydryl ethers having antihistaminic and tranquillizing properties. This antagonism could not be explained at that time Further investigation of the problem by DE WAART (1958b) and ERNSTING *et al* (1960) brought to light that various drugs influenced release of bound amino acids in metabolizing rat brain slices. ERNSTING *et al* (1961) suggested that membrane structures were involved with this type of stimulation

In the past decades the numerous reports made on action of phenothiazines on enzymes described inhibitory effects, only DESCI (1957) mentioned incidentally a stimulation of ATP-ase by Multergan in rat brain homogenates

Recently LOVTRUP (1965) assumed that the activation inhibition phenomenon of different concentrations of chlorpromazine on ATP-ase and succinate dehydrogenase was understandable in terms of interference with the structure of the mitochondrion membrane GUTH *et al* (1964 1965) emphasized the significance of the effect that the phenothiazines exert on membranes where these drugs are sufficiently concentrated in the body They suggested that changes in membrane permeability may be the underlying mechanism of action of phenothiazines in tranquillization

TABLE I

p-nitrophenylphosphatase activity in fractions of rat tissue.

	pH **	Brain	Liver	Kidney
homogenate	9.6–10.0	2.8–4.2	6.0– 8.0	108 –120
homogenate	7.2– 7.4	6.2–6.8	13.2–16.7	23.5– 27.0
nuclei	7.2– 7.4	0.3–0.5	2.3– 3.2	3.0– 4.5
crude mitochondria	7.2– 7.4	1.6–1.8	2.8– 3.7	6.1– 9.5
microsomes	7.2– 7.4	1.0–1.2	2.3– 3.1	4.9– 6.0
supernatant	7.2– 7.4	1.9–2.2	4.2– 5.5	6.6– 8.4

T   figures in the table reflect the range of the activities collected from three or more experiments.

expressed in µmole p-nitrophenol formed per 100 mg dissected tissue in 60 min at 37 °C.

** glycine buffer pH 9.6–10.0
Tris buffer pH 7.2 7.4

With the isolation procedure used, the majority of the activity is found in the particulate fractions. In order to reduce the laborious work of investigating the effect of substances on all of the particulate fractions of the different organs separately we screened a number of drugs primarily in homogenate preparations. In consideration of publications of SEEMAN (1963), BILZI (1965) and ZOGRAFI (1966) on surface tension-lowering properties of the phenothiazines we added for comparative purposes a few non-ionic detergents to the series.

We designed our experiments in such a way that always both normal and drug-influenced activity of the pooled organs from the same animals were taken in one run. Putting the normal value of each fraction at 100% we could show in the table the effect of drugs most reliable in mutual comparison. For reason of uniformity the effects of mM concentrations were presented. Optimal concentration could differ however for instance Thiazinamium shows maximum effect at 3 mM and Chlorpromazine at 1 mM.

We have not listed compounds, which had no stimulating effects like barbiturates, procaine, iproniazide acetylcholine and others. Also we have not presented data from kidney tissue by lack of

was not treated further All sedimented fractions were carefully resuspended in 0 25 M sucrose by hand homogenization Suspension concentrations of the particulate fraction were adapted to experimental conditions

Determinations of pnpp-activity were performed in 10 ml incubation mixture which consisted of 0 5 ml tissue preparation 7 ml 0 20 M Trihydroxymethylaminomethane buffer pH 7 * ('Tris Koch Light) containing 1 mM $MgCl_2$ and 2 5 ml 4 mM *p*-nitrophenylphosphate-Na (British Drug Houses)

After a 60 min incubation period at 37 C in a Dubnow shaker 1 ml was taken from the reaction fluid and pipetted into 11 ml 0 02 M NaOH If turbidity occurred (as in the presence of chlorpromazine) this alkaline solution was centrifuged

Extinction of the yellow colour of *p*-nitrophenol was measured in a Vitatron photometer U C 100 with a 415 nm interference filter Activity of the enzyme splitting *p*-nitrophenylphosphate was expressed in μ mole *p*-nitrophenol formed in 1 h at 37 C by preparations derived from or corresponding to 100 mg dissected wet tissue (μ mole/100 mg)

RESULTS AND DISCUSSION

Although alkaline phosphatase (E C 3 1 3 1) in the body is characterized by its activity in alkaline milieu *in vitro* it is presumed to work *in vivo* predominantly in physiological pH range In addition MORTON (1957) and SAEV (1964) observed that the pH-optimum of alkaline phosphatase in tissues shifts to the physiological range when substrate concentrations are low and approach physiological levels The pH curves of the *p*-nitrophenylphosphatase (pnpp) activity in brain liver and kidney exhibit different characteristics (unpublished results, authors) In contrast to a distinct alkaline pH optimum of pnpp in kidney tissue we could not demonstrate any pronounced pH optimum in brain tissue From maximal activity in acid milieu the pH curve for brain tissue declined slightly towards the alkaline side Owing to the noteworthy results in the physiological pH range, especially in brain we focussed our investigations on this pH region From 31 experiments, the range of pnpp activities of homogenates and subcellular fractions are shown in Table I

phosphatases. In our opinion it is more probable that the results indicate that the pnpp enzyme in brain tissue has a different structural environment than in liver and kidney tissue In consequence of this different incorporation of the membranes of various tissues we can expect a particular effect of drugs, which are now presumed to influence the structure of the membranes. Those compounds showing a stimulatory effect on the splitting of *p*-nitrophenyl phosphate in brain homogenates were selected for further investigation of their effect on the same enzyme activity in particulate cell fractions. Little or no effect of these substances was observed in nuclear and supernatant fractions.

Therefore we concentrated on the crude mitochondrial and microsomal fractions.

From Table 2 we learn that the particulate fractions of the brain showed a greater stimulating effect than that obtained in homogenates.

Even those substances having a small effect on liver homogenate showed a greater effect on the mitochondrial fraction.

Although we may presume a similar mechanism of action of the drugs in liver-mitochondria with respect to the increased activity of its bound pnpp enzyme it is clearly demonstrated that in particular observed the drug-action is at the subcellular particle of the brain cell.

The question arose whether this promoted enzyme activity was mere release of concealed enzyme from the membrane containing systems involved, or a revealing of additional active sites of the embedded enzyme concomitantly with a better penetration of the substrate This problem could be solved with the aid of detergents. The solubilizing properties of detergents are based on the fragmentation of the membrane Bound enzymes become partially or fully released by treatment with detergents and are detected in the supernatant after centrifugation. With this possibility in mind, we pre-incubated homogenates or crude mitochondria suspensions of cerebral hemispheres in 0.2 M Tris buffer pH 7.. (volume ratio 3 7) without substrate during 30 min at 37 °C, with addition of 1 mM Chlorpromazine or 0.1% Sterox SE. After the pre-incubation period particles were sedimented at 105 000 × g for 60 min. Pnpp-activity of sediment and supernatant was determined in the usual way

significant action From the table we learn that drug action is strongest in brain tissue

TABLE 2

Effect of various drugs and detergents on p nitrophenylphosphatase activity in particulate fractions*

	Brain			Liver		
	Hom	Mit	Micr	Hom.	Mit.	Micr
None	100	100	100	100	100	100
Promethazine	175	190	202	105	125	72
Thiazinamium	170	217	243	118	139	105
Chlorpromazine	167	189	213	118	108	64
Levomepromazine	103	166	144	[illegible]	136	145
Imipramine	101	[illegible]	207	110	129	82
Neo-Antergan	116	131	118	111	123	125
Diphenhydramine	[illegible]	133	[illegible]	111	128	124
Neobenodine	150	162	175	[illegible]	149	128
Orphenadrine	151	154	171	13	—	—
Chlorcyclizine	170	198	[illegible]	108	141	75
Sterox 0 1%	157	107	130	[illegible]	—	—
Triton X 100 0 1%	157	104	121	122	125	76
Digitonine 0 1%	155	144	118	105	12	90

* Activities are determined in Tris buffer pH 7.2 at 37 °C.
* Each of the fractions is referred to its control, which = 100%. All drug concentrations are 2 mM.

In the phenothiazine group both promethazine and thiazinamium, known for their strong antihistaminic properties show the same level of activity as chlorpromazine

At the bottom of the list the figures of the action of three detergents on pnpp activity showed that the best stimulating effect in the organs tested was found in brain homogenate or in other words the solubilizing and fragmentizing action of detergents on membrane bound enzymes is demonstrated in brain tissue most effectively Although we found in a parallel investigation (un published) that brain liver and kidney had different iso-enzyme-patterns of the non-specific alkaline phosphatase and also different pH-curves of the pnpp we think it is unlikely that the differential drug-effects in the tissues merely stem from different kinds of

remaining attached to the membrane. This effect of drugs is reversible, as we observed from pre-incubation experiments.

In order to know whether the effect was restricted to pnpp alone we carried out similar experiments with glutamic acid decarboxylase (EC.4 1 1 15) and found corresponding results.

Presumably this applies to other membrane-bound enzymes as well.

In conclusion the *in vitro* experiments indicated that the stimulating effect of phenothiazine-drugs among others is due to their membrane altering properties rather than to a direct interaction with the enzymes involved.

ABSTRACT

In brain and liver of the rat p-nitrophenylphosphatase activity was determined at physiological pH range.

Phenothiazines and several other drugs increased the p-nitrophenylphosphatase activity bound to subcellular particles, especially of brain.

Brain tissue which was pre-incubated with effective drugs maintained the enzymes in a bound state in contrast to detergents which solubilized them to great extent.

In discussion the site of action of the stimulation phenomenon was ascribed to alteration of the membrane architecture which consequently revealed additional active sites of the bound enzyme.

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TABLE 3

Distribution of bound/solubilized p-nitrophenylphosphatase activity after pre-incubation.

Fraction	30 min pre incubated at 37 °C	treatment after pre-incubation sediment 105.000 × g	supernatant 105.000 × g
Homogenate	no addition	78 %	2[illegible] %
	1 mM Chlorpromazine	81	19
	0.1 % Sterox SE	43	57
Crude mitochondria	no addition	73 %	[illegible]7 %
	1 mM Chlorpromazine	87	13
	0.1 % Sterox SE	49	51

Figures represent % distribution of recovered activity in fractions.

When pre-treated with Chlorpromazine (Table 3) the ratio bound/soluble phosphatases showed no difference from control ratios. The detergent on the contrary solubilized the enzyme to a large extent.

These observations reflect that although drug and detergent both increase the pnpp-splitting activity their mechanisms of action are basicly different. Probably the drugs in question alter the membrane structure without damaging it causing an unmasking of the active sites and better access of substrate to active sites on the bound enzyme.

A mere stimulation however should be reflected in a changed Michaelis constant *Km*. From experiments according to the method of Lineweaver and Burk we calculated the apparent $Km = 9 \times 10^{-4}$ M both for reference as for drug influenced brain homogenates. With no change in *Km* value although an increase of maximum rate was noticed the phenomenon must be due to integrity alteration of the membrane which made the substrate better accessible to concealed active sites. We intentionally avoid introduction of the factor of membrane permeability as our experiments were not designed to measure transport across membranes. Under influence of the drugs the bound pnpp enzyme achieves full expression while

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LIPOLYTIC ACTIVITIES OF A PARTIALLY PURIFIED ENZYME OF THE ELASTASE COMPLEX [1])

BY

W. A. LOEVEN [2])

1 INTRODUCTION

Recently the release of the carbohydrate moieties from alkali-treated and acid-treated elastin was studied by incubating elastin with various enzymes of the elastase complex prepared by means of a column chromatographic technique (LOEVEN 1965a, b). The results of these experiments agreed with data in the literature concerning the heterogeneity of the carbohydrate moieties in elastin (e.g. BANGA and BALÓ 1956 GOTTE et al., 1963 MORET et al 1964 WALFORD et al., 1959 1961)

The results of the investigation on the release of lipid like material during the incubation of elastin with these enzymes were however in disagreement with the opinions of BANGA and BALÓ (196) HALL (1961) and SAXL (1961 1962) who believe that the mucolytic elastase component has a lipolytic character rather than a true mucolytic one and should be called elastolipoproteinase (HALL, 1964) In a preliminary note we have already mentioned that none of the components of the elastase complex used in our experiments showed any lipolytic activity (LOEVEN 1965b).

It appears, however that pancreas powder contains still another enzyme one that has esterolytic activity on "lipid substrates

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[1]) This article is Part IV of the series on "Elastolysis" Part I appeared in the Acta Physiol. Pharmacol. Neerl. 9 (1960) 473–600; Part II in the Proceedings of the NATO-Conference on the Structure and Function of Connective and Skeletal Tissues (St. Andrews, Scotland, 15–25 June, 1964; Butterworths Publ. London, 1965) pp. 109–116; Part III in the Acta Physiol. Pharmacol. Neerl. 13 (196.) 278–303.

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in many respects a combination of that of the elastomucases Em I and Em-S (for a more detailed survey of these components of the elastase complex, see LOEVEN 1965a, b c).

Recently it was found that with this chromatographic technique other fractions with a synergistic effect on the activity of elastoproteinase can also be eluted from the column when a stepwisely increasing salt gradient with NaCl is introduced. Fig 1 shows typical elution diagrams of crude elastoproteinase + Em I – rich and of crude elastomucase (Em-S) – rich samples prepared from an acetate extract of pancreas powder (these two crude samples are called AEI and AES, respectively following the terminology of HALL and CZERKAWSKI, 1959).

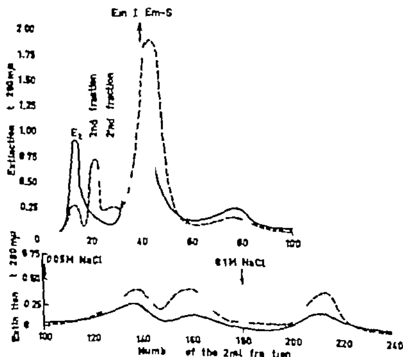


Fig. 1

Chromatography of 500 mg crude AEI preparation (containing elastoproteinase (E_a) and elastomucase (Em I); ———) and 500 mg crude AES preparation (containing elastomucase Em-S and the so-called second fractions; – – –), on DEAE-Sephadex column developed with a Na_2CO_3-HCl buffer ($\Gamma/2 = 0.135$; pH = 8.8) and a stepwisely increased NaCl gradient.

and releases lipid like material from acid treated and crude elastin and aorta powder in this way showing a synergistic effect on the activity of elastoproteinase

Some preliminary results of the lipolytic activity of this partially purified enzyme (which seems to differ from pancreatic lipase phospholipase A, and aliesterases) will be presented in this paper

2. MATERIAL AND METHODS

2.1 Substrates

Acid and alkali treated elastin were prepared from bovine ligamentum nuchae as already described in detail elsewhere (Loeven 1960)

The calf aorta powder used in some experiments was prepared from fresh material obtained from the slaughterhouse The aortae were dried with acetone minced in a power-driven mincing machine (Poppink, Amsterdam) using sieves with holes of 1 0 0 5 and 0 2 mm diameter extracted with 0 9% NaCl until the extract gave a negative protein test with the biuret method dried with acetone and ethylether and finally minced again through a 0 2 mm sieve

The human serum was a pooled serum sample kindly placed at our disposal by the Bloodbank of the University Hospital of Leiden

The other substrates were commercial products Tween 20 (Koch-Light Labor Ltd Colnbrook England) *Ediol* (*Riker* Laboratories, Northridge California U S A) glycerol trioleïn (The British Drughouses Ltd Poole England) β naphthyllaurate (Nutritional Biochemicals Corp Cleveland Ohio U S A.)

2 2 Preparation of the enzymes and some of their characteristics

Elastoproteinase and the two elastomucases (Em I and Em-S) were purified enzyme samples isolated from pancreas powder (Viokase from Viobin Corp Monticelli Ill U.S A. or powder from Organon N V Oss, The Netherlands) by means of column chromatography using DEAE-Sephadex as packing material

The behaviour of two other enzyme fractions which shows mucolytic activity – the so-called 2nd fractions – appears to be

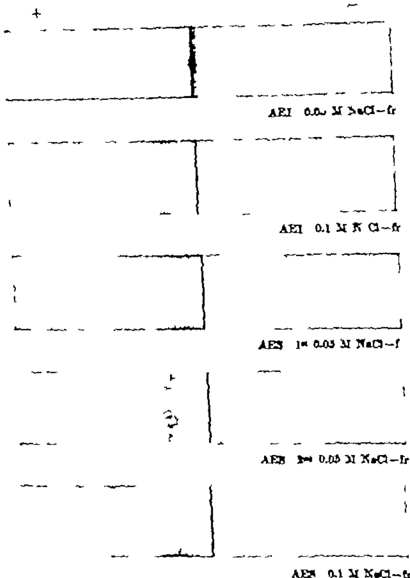


Fig. –

Paper electropherograms of the fractions eluted from the column of Fig. 1 after the addition to the buffer solution of 0.05 and 0.1 M NaCl (Spinco apparatus; Veronal buffer of $\Gamma/2 = 0.075$, pH = 8.7; constant current of 14 mA; average voltage of 180 V; running period of 11 hrs; paper strips stained with amido black 10B; Whatman 3 MM paper).

Nearly all the enzyme fractions that can be eluted with the Na_2CO_3-HCl buffer (pH 8 8 $\Gamma/2 = 0$ 13) give protein bands at the cathodic side in the paper electropherogram (elastoproteinase running faster than Em I whereas Em-S gives a more diffuse protein band near the line of application see LOEVEN 1965a, b) Only the small fraction that can be eluted with the buffer after the elastomucase-containing fraction gives a rather diffuse protein band lying just at the anodic side of the line of application.

This small fraction present in both crude enzyme samples, has not been studied extensively but the results obtained so far suggest the presence of partially denatured but still active elastomucase (see Table 1) The same conclusion may be drawn from the preliminary experiments with the fractions eluted from the column after the addition of 0 05 M NaCl to the buffer solution Fig 9 shows the paper electropherograms of the various fractions eluted after the addition of a salt gradient to the carbonate buffer The two fractions eluted with 0 05 M NaCl in the buffer and obtained from AES samples show two protein bands at the anodic side of the line of application (the paper strips are stained with amido black 10B) When chromatographing AEI samples we found that the 0 05 M NaCl fraction (sometimes present as two peaks largely overlapping each other in the elution diagram) contains material that is nearly immobile during paper electrophoresis and shows slight lipase activity when its activity is tested against a specific synthetic substrate for lipase namely β-naphthyllaurate in the presence of sodium taurocholate (see Table 2)

The behaviour of the fractions eluted from the column when the NaCl gradient is increased to 0 1 molar will be discussed in detail later in this paper Here we will only mention that the 0 1 M NaCl fraction from crude AEI samples shows two protein bands in the paper electropherogram one band upon the line of application (containing pancreatic lipase see Table 2) and one band of a protein that behaves as a serum β-globulin and must be considered to be the elastolipoproteinase (see below)

The 0 1 M NaCl fraction from crude AES samples also consists of two protein components the β-globulin like protein mentioned above (= elastolipoproteinase) and an α_2-globulin like protein also present in the 0 05 M NaCl fractions from AES samples.

Table 1 shows the elastolytic and general proteolytic activities

[illegible]	0.05 M NaCl fr from AEI (3 samples)	0.10 M NaCl fr from AEI (4 samples)	0.10 NaCl fr from AES (4 samples)	Lipase (Merck)	experimental conditions
[illegible]	0.10–0.14	0.18–0.31	0.31–0.40	1.56	1% Tween 20 in (5% albumin + 0.05 M $(NH_4)_2SO_4$) pH 6.7
	[illegible]	0.3	0.29–0.52	2.39	pH 7.[illegible]
			[illegible]	2.10	method of DESNUELLE et al. (1955)
			[illegible]	5.33	pH 8.6 method of HOOD et al. (1962)
			0.05–0.12	6.16	pH 7.2
			0.03–0.08	2.50	pH 8.6
			0.06–0.13	2.24	pH 7.2
					3 ml. serum + 1 ml enzyme (in 0.9% NaCl)
		0.01–0.02	0.06–0.14	0.46	pH 8.6
	0	0.02–0.04	0.09–0.21	0.59	pH 7.2
					SELIGMAN and NACHLAS (1950)
β-naphthyl laurat	0.00–0.03	0.21–0.62	0.00–0.01	0.94	without taurocholat in system
	0.15–0.33	0.61 1.17	0.01–0.02	4.64	with 10 mM-taurocholate in system

TABLE I

The elastolytic and proteolytic activities as well as the synergistic effects on the activity of elastoproteinase of the various fractions containing components of the elastase complex and obtained from crude pancreatic extracts by means of DEAE-Sephadex column chromatography

enzyme fraction	origin	activity on alkali treated elastin at pH 8.7 a)			activity on acid treated elastin pH 7.2 a)		proteolytic activities on			number of enzyme samples investigated
		enzyme conc. (mg)	elastin dissolved (mg)	synergistic effect (%)	elastin dissolved (mg)	synergistic effect (%)	casein b)	BAEE c)	TEE d)	
elastoproteinase	crude AEI	0.1	11.6–18.3	–	6.5–9.7	–	42–46	0.4–4.8	0.1–0.6	5
elastomucase (Em I)	crude AEI	0.25	7.2–11.7	70–140	6.3–10.5	120–185(max.)	11–23	0.1–0.3	4.0–4.5	5
last buffer fr	crude AEI	1.0	7.1–7.8	20–37	7.8–8.5	63–135	64–72	–	–	2
0.05 M NaCl fr	crude AEI	2.0	6.7–7.1	20–40	6.2–6.5	50–62	117–138	0.0–0.1	0.8–1.2	3
0.10 M NaCl fr	crude AEI	3.0	2.3–4.5	0–5	1.1–3.5	30–46	210–2"2	0.0–0.1	0.1–0.4	4
2nd buffer fr	crude AES	0.5	6.1–7.4	60–66	4.0–7.4	45–85 (max.)	45–74	0.3–0.7	1.0–2.3	3
elastomucase (Em-S)	crude AES	1.0	4.0–7.2	35–54	2.4–3.8	60–105(max.)	76–104	0.1–0.4	2.9–7.6	6
last buffer fr	crude AES	2.0	2.4–2.9	17–23	1.9–2.3	55–64	68	–	–	2
0.05 M NaCl (total) fr	crude AES	3.0	1.8–4.4	0–20	1.6–3.9	30–45	78–86	0.1–0.2	3.3–4.0	3
0.10 M NaCl fr	crude AES	3.0	1.5–4.7	0–10	1.3–4.1	35–65	115–208	0.0–0.1	1.0–3.8	4
trypsin f)							62–87	33.0–39.4	0	
chymotrypsin f)							10–16	0.0–0.1	6.8–10.0	

a) The system consists of 50 mg substrate in 10 ml borate buffer. Incubation time 3 hrs at 37 °C. With both kinds of substrate the same enzyme concentration is used. For further details of the method used see LOEVEN (1960, 1962a).

b) The casein digestion was measured according to Kunitz (see LASKOWSKI, 1955) and the enzymic activity expressed as the amount of enzyme in µg needed to solubilize an amount of casein such that an extinction of .25 is registered at 280 mµ.

) The activity on N benzoyl L-arginine ethylester was calculated as the amount of BAEE (in µ moles) hydrolysed by 1 mg enzyme (SCHWERT and TAKENAKA, 1955).

d) The activity on L-tyrosine ethylester was calculated in the same way as for BAEE.

f) [illegible] preparation from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. (twice crystall., salt free)

[illegible] preparations AEI and AEII as compared to the activity of pancreatic lipase on the same substrates. The activity is expressed as μmoles fatty acid liberated by 1 mg enzyme fraction from 1 ml substrate in 1 hr (Dole [illegible] Meinertz method, 1960); in the case of β-naphthyl-laurate the activity is expressed as mg β-naphthol/mg enzyme/hr

enzyme / substrat	0.03 M NaCl fr from AEI (3 samples)	0.10 M NaCl fr from AEI (4 samples)	0.10 NaCl fr from AEII (4 samples)	Lipase (Merck)	experimental conditions
Tween 20	0.10–0.14	0.18–0.21	0.21–0.40	1.56	1% Tween 20 in (5% albumin + 0.05 M $(NH_4)_2SO_4$) pH 8.7
	0.18–0.23	0.33–0.68	0.29–0.53	2.39	pH 7.2
triolein	0.06–0.10	0.30–0.53	0.04–0.07	2.10	method of Dervichian et al. (1955)
ediol (non-activated)	0.06–0.13	0.16–0.23	0.02–0.09	5.33	pH 8.6 method of Hood et al. (1962)
	0.10–0.17	0.21–0.30	0.05–0.12	9.16	pH 7.2
(activated)	0.04–0.10	0.10–0.18	0.03–0.08	2.50	pH 8.6
	0.06–0.11	0.14–0.26	0.06–0.13	3.24	pH 7.2
human serum (pooled)	0	0.01–0.02	0.06–0.14	0.46	2 ml serum + 1 ml enzyme (in 0.9% NaCl) pH 8.6
	0	0.02–0.04	0.09–0.21	0.59	pH 7.2
β-naphthyl laurate	0.00–0.03	0.21–0.62	0.00–0.01	0.94	Seligman and Nachlas (1950) without taurocholate in system
	0.15–0.23	0.51–1.17	0.01–0.03	4.64	with 10 mM-taurocholate in system

TABLE 1

The elastolytic and proteolytic activities as well as the synergistic effects on the activity of elastoproteinase of the various fractions containing components of the elastase complex and obtained from crude pancreatic extracts by means of DEAE-Sephadex column chromatography

enzyme fraction	origin	activity on alkali treated elastin at pH 8.7a): enzyme conc. (mg)	activity on alkali treated elastin at pH 8.7a): elastin dissolved (mg)	activity on alkali treated elastin at pH 8.7a): synergistic effect (%)	activity on acid treated elastin pH 7.2a): elastin dissolved (mg)	activity on acid treated elastin pH 7.2a): synergistic effect (%)	proteolytic activities on: caseinb)	proteolytic activities on: BAEEc)	proteolytic activities on: TEEd)	number of enzyme samples investigated
elastoproteinase	crude AEI	0 1	11 6–18.3	–	6.5– 9 7	–	42–46	0 4– 4.8	0 1– 0 6	5
elastomucase (Em I)	crude AEI	0.25	7.2–11 7	70–140	6.3–10.5	120 185(max.)	11–22	0 1– 0.3	4.0– 4.5	5
last buffer fr	crude AEI	1 0	7 1– 7 8	20–37	7.8– 8.5	63–135	64–72	–	–	2
0 05 M NaCl fr	crude AEI	2.0	6.7– 7 1	20–40	6.2– 6.5	50–62	117–138	0 0– 0 1	0.8– 1.2	3
0.10 M NaCl fr	crude AEI	3 0	2.3– 4.5	0–5	1 1– 3.8	30–46	210–27.	0 0– 0 1	0 1– 0 4	4
2nd buffer fr	crude AE8	0.5	6.1– 7 4	60–66	4 0– 7 4	45–85 (max.)	45–74	0.3– 0 7	1 9– 2.3	3
elastomucase (Em-8)	crude AE8	1 0	4.0– 7.2	35–54	2 4– 3.8	60–105(max.)	70–104	0 1– 0 4	2.9– 7 6	6
last buffer fr	crude AE8	2 0	2.4– 2.9	17–23	1.9– 2.3	55–64	68	–	–	2
0 05 M NaCl (total) fr	crude AE8	3 0	1.8– 4 4	0–20	1 6– 3.9	30–45	78–86	0 1– 0.2	3.3– 4 0	3
0 10 M NaCl fr	crude AE8	3 0	1.5– 4.7	0–10	1.3– 4.1	35–65	115–208	0 0– 0.1	1 0– 3 8	4
trypsin 1)							62–87	33.0–39 4	0	
chymotrypsin 2)							10–16	0.0– 0 1	0.8–10 0	

a) The system consists of 50 mg substrate in 10 ml borate buffer Incubation time 3 hrs at 37 °C. With both kinds of substrate the same enzyme concentration is used. For further details of the method used see LOEVEN (1960 1963a)

b) The casein digestion was measured according to Kunitz (see LASKOWSKI, 1955) and the enzymic activity expressed as the amount of enzyme in μg needed to solubilize an amount of casein such that an extinction of .25 is registered at 280 $m\mu$.

c) The activity on N benzoyl L-arginine ethylester was calculated as the amount of BAEE (in μ moles) hydrolysed by 1 mg enzyme (SCHWERT and TAKENAKA, 1955)

d) The activity on L-tyrosine ethylester was calculated in the same way as for BAEE.

1) Preparation from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. (twice crystall., salt free)

[illegible] *preparations AB* [illegible] *AMP as compared to the activity of pancreatic lipase on the same substrates. The activity is expressed as µeq. fatty acid liberated by* [illegible] *mg enzyme fraction from 1 ml substrate in 1 hr (DOLE AND MEINERTZ's method, 1960)* [illegible] *on the case of β-naphthyl* [illegible] *the activity is expressed as mg β-naphthol/mg enzyme/hr*

substrate \ enzyme	0.05 M NaCl fr from AEI (3 samples)	0.10 M NaCl fr from AEI (4 samples)	0.10 NaCl fr from AES (4 samples)	Lipase (Merck)	experimental conditions
Tween 20	0.10–0.14	0.18–0.21	0.21–0.40	1.56	1% Tween 20 in (5% albumin+0.05 M $(NH_4)_2SO_4$) pH 6.7
	0.19–0.22	0.22–0.58	0.29–0.53	2.19	pH 7.2
triolein	0.06–0.10	0.20–0.43	0.04–0.07	2.10	method of DIRSTINE et al. (1965)
ediol (non-activated)	0.06–0.12	0.18–0.22	0.02–0.09	5.33	pH 8.6 method of HOOD et al. (1962)
	0.10–0.17	0.21–0.30	0.05–0.12	4.16	pH 7.2
(activated)	0.04–0.10	0.10–0.18	0.03–0.09	2.50	pH 8.6
	0.06–0.11	0.14–0.26	0.06–0.13	3.24	pH 7..
human serum (pooled)					3 ml serum+1 ml enzyme (in 0.9% NaCl)
	0	0.01–0.02	0.06–0.14	0.46	pH 8.6
	0	0.03–0.04	0.09–0.21	0.59	pH 7.2
β-naphthyl-laurate					SELIGMAN and NACHLAS (1950)
	0.00–0.03	0.31–0.62	0.00–0.01	0.94	without taurocholate in system
	0.15–0.23	0.51–1.17	0.01–0.02	4.54	with 10 mM-taurocholat in system

TABLE 1

The elastolytic and proteolytic activities as well as the synergistic effects on the activity of elastoproteinase of the various fractions containing components of the elastase complex and obtained from crude pancreatic extracts by means of DEAE-Sephadex column chromatography

enzyme fraction	origin	activity on alkali treated elastin at pH 8.7ᵃ)			activity on acid treated elastin pH 7.2ᵃ)		proteolytic activities on			number of enzyme samples investigated
		enzyme conc. (mg)	elastin dissolved (mg)	synergistic effect (%)	elastin dissolved (mg)	synergistic effect (%)	casein[b])	BAEE)	TEE[d])	
elastoproteinase	crude AEI	0 1	11 6–18.3	–	6.5– 9 7	–	42–46	0 4– 4.8	0 1– 0 6	5
elastomucase (Em I)	crude AEI	0.25	7.2–11 7	70–140	6.3–10.5	120 185(max.)	11–22	0 1– 0.3	4 0– 4.5	5
last buffer fr	crude AEI	1 0	7 1– 7.8	20–37	7.8– 8.5	83 135	64–72	–	–	2
0.05 M NaCl fr	crude AEI	2.0	6.7– 7 1	20–40	6.2– 6.5	50–62	117–138	0 0– 0 1	0.8– 1.2	3
0 10 M NaCl fr	crude AEI	3 0	2.3– 4.5	0–5	1 1– 3.8	30–46	210–272	0 0– 0 1	0 1– 0 4	4
2nd buffer fr	crude AES	0.5	6 1– 7 4	60–66	4 0– 7 4	45–85 (max.)	45–74	0.3– 0 7	1 9– 2.3	3
elastomucase (Em-S)	crude AES	1 0	4.0– 7.2	35–54	2.4– 3.8	60–106(max.)	76–104	0 1– 0 4	2.0– 7 6	6
last buffer fr	crude AES	2.0	2 4– 2.9	17–23	1 9– 2.3	55–64	68	–	–	2
0 05 M NaCl (total) fr	crude AES	3.0	1 8– 4.4	0–20	1 6– 3 9	30–45	78–86	0 1– 0.2	3.3– 4.0	3
0 10 M NaCl fr	crude AES	3.0	1.5– 4.7	0–10	1.3– 4.1	35–65	115–208	0.0– 0.1	1 0– 3.8	4
trypsin [f])							62–87	33 0–39 4	0	
chymotrypsin [f])							10–16	0 0– 0 1	0 8–10 0	

a) The system consists of 50 mg substrate in 10 ml borate buffer Incubation time 3 hrs at 37 °C. With both kinds of substrate the same *enzyme concentration is used. For further details* of the method used see LOEVEN (1960 1963a)

b) The casein digestion was measured according to Kunitz (see LASKOWSKI, 1955) and the enzymic activity expressed as the amount of enzyme in μg needed to solubilize an amount of casein such that an extinction of .25 is registered at 280 mμ.

c) The activity on N benzoyl L-arginine ethylester was calculated as the amount of BAEE (in μ moles) hydrolyzed by 1 mg enzyme (SCHWERT and TAKENAKA, 1955).

d) The activi y on L-tyrosine ethylester was calculated in the same way as for BAEE.

— Preparation rom the Nutritional Biochemicals Corp., Cleveland Ohio, U.S.A. (wice crystall, sal free)

[illegible] preparations [illegible] as compared to the activity of pancreatic lipase on the same substrates. The activity is expressed as [illegible] fatty acid liberated by 1 mg enzyme fraction from 1 ml substrate in 1 hr (DOLE and MEINERTZ method, 1960). In the case of β-naphthyl-laurate the activity is expressed as mg β-naphthol/mg enzyme/hr

substrate \ enzyme	0.06 M NaCl fr from AEI (3 samples)	0.10 M NaCl fr from AEI (4 samples)	0.10 NaCl fr from AE8 (4 samples)	Lipase (Merck)	experimental conditions
Tween 20	0.10–0.14 0.16–0.22	0.16–0.31 0.33–0.68	0.31–0.40 0.39–0.53	1.56 2.39	1% Tween 20 in (5% albumin+0.05 M $(NH_4)_2SO_4$) pH 8.7 pH 7.2
triolein	0.06–0.10	0.30–0.53	0.04–0.07	2.10	method of DESNUELLE et al. (1955)
olive oil (non-activated)	0.06–0.12 0.10–0.17	0.18–0.33 0.21–0.30	0.02–0.09 0.06–0.12	5.33 6.16	pH 8.6 method of HOOD et al. (1962) pH 7.2
(activated)	0.04–0.10 0.06–0.11	0.10–0.15 0.14–0.26	0.03–0.06 0.06–0.13	2.50 2.24	pH 8.6 pH 7.2
human serum (pooled)	0 0	0.01–0.02 0.02–0.04	0.06–0.14 0.09–0.21	0.46 0.59	2 ml serum+1 ml enzyme (in 0.9% NaCl) pH 8.6 pH 7.2
β-naphthyl laurate	0.00–0.03 0.16–0.22	0.21–0.62 0.51–1.17	0.00–0.01 0.01–0.02	0.94 4.54	SELIGMAN and NACHLAS (1950) without taurocholat in system with 10 mM taurocholate in system

TABLE 1

The elastolytic and proteolytic activities as well as the synergistic effects on the activity of elastoproteinase of the various fractions containing components of the elastase complex and obtained from crude pancreatic extracts by means of DEAE-Sephadex column chromatography

enzyme fraction	origin	activity on alkali treated elastin at pH 8.7[a])			activity on acid treated elastin pH 7.2[a])		proteolytic activities on			number of enzyme samples investigated
		enzyme conc. (mg)	elastin dissolved (mg)	synergistic effect (%)	elastin dissolved (mg)	synergistic effect (%)	casein[b])	BAEE[c])	TEE[d])	
elastoproteinase	crude AEI	0.1	11.6–18.3	–	6.5– 9.7	–	42–46	0.4– 4.8	0.1– 0.6	5
elastomucase (Em I)	crude AEI	0.25	7.2–11.7	70–140	6.3–10.5	120 188(max.)	11–22	0.1– 0.3	4.0– 4.5	5
last buffer fr	crude AEI	1.0	7.1– 7.8	20–37	7.8– 8.5	63 135	64–72	–	–	2
0.05 M NaCl fr	crude AEI	2.0	6.7– 7.1	20–40	6.2– 6.5	50–62	117–138	0.0– 0.1	0.8– 1.2	3
0.10 M NaCl fr	crude AEI	3.0	2.3– 4.5	0–5	1.1– 3.8	30–46	210–272	0.0– 0.1	0.1– 0.4	4
2nd buffer fr	crude AES	0.5	6.1– 7.4	60–66	4.0– 7.4	45–85 (max.)	45–74	0.3– 0.7	1.9– 2.3	3
elastomucase (Em-S)	crude AES	1.0	4.0– 7.2	35–54	2.4– 3.8	60–105(max.)	76–104	0.1– 0.4	2.9– 7.6	6
last buffer fr	crude AES	2.0	2.4– 2.9	17–23	1.9– 2.3	55–64	68	–	–	2
0.05 M NaCl (total) fr	crude AES	3.0	1.8– 4.4	0–20	1.6– 3.9	30–45	78–86	0.1– 0.2	3.3– 4.0	3
0.10 M NaCl fr	crude AES	3.0	1.5– 4.7	0–10	1.3– 4.1	35–65	115–208	0.0– 0.1	1.0– 3.8	4
trypsin [e])							62–87	33.0–39.4	0	
chymotrypsin [e])							10–16	0.0– 0.1	6.8–10.0	

a) The system consists of 50 mg substrate in 10 ml borate buffer Incubation time 3 hrs at 37 °C With both kinds of substrate the same *enzyme concentration is used. For further details of the method used see* LOEVEN (1960 1963a)

b) The casein digestion was measured according to Kunitz (see LASKOWSKI 1955) and the enzymic activity expressed as the amount of enzyme in μg needed to solubilize an amount of casein such that an extinction of 25 is registered at 280 mμ

c) The activity on N benzoyl L-arginine ethylester was calculated as the amount of BAEE (in μ moles) hydrolysed by 1 mg enzyme (SCHWERT and TAKENAKA, 1955)

d) The activity on L-tyrosine ethylester was calculated in the same way as for BAEE.

e) Preparation from the Nutritional Biochemicals Corp., Cleveland Ohio, U.S.A. (twice crystall. salt free)

Table 2 gives the results obtained with the enzyme fractions indeed showing esterolytic activities.

The following enzyme fractions are completely devoid of any esterolytic activity on the substrates used elastoproteinase the elastomucases Em I and Em-S the 2nd buffer fractions, the last buffer fractions, and the 0 05 M NaCl fractions prepared from crude AES samples. The Table shows that the 0 05 M NaCl and especially the 0 10 M NaCl fractions isolated from crude AEI samples are still contamined with lipase (activity on β-naphtyllaurate in the presence of taurocholate) whereas the 0 10 M NaCl fraction from crude AES samples may be considered to be free of lipase Table 3 summarizes the data concerning the effect of NaCl and some organic substances on the esterolytic activity of the 0 1 M NaCl fraction from AES.

TABLE 3

Effect of some organic compounds and NaCl on the esterolytic activity of the 0.1 M NaCl fraction isolated from crude elastase preparation AES using as substrates Tween 20 and normal human serum (activity expressed µ equiv fatty acid released from 1 ml system/mg enzyme/hr)

reagent added	activity (as percentage of control) on: 1% Tween 20 (+ 5% albumin)		human serum	
	pH 7.3	pH 8.7	pH 7.3	pH 8.7
heparin (0.1 mg/ml system)	91 %	100%	76%	49%
cysteine (10 mM in system)	82	99%	—	—
Protamine (0.25 mg/ml system)	99%	95%	81%	27%
Na-taurocholate (0.1% in system)	106%	109%	—	—
Na-deoxycholate (0.2% in system)	130%	106%	58%	79%
NaCl (1 M in system)	72%	95%	45%	76%

of all these eluted enzyme fractions and their activation activities on the elastolysis by elastoproteinase (i.e. their synergistic effect on the activity of elastoproteinase). The most striking results are those obtained with the 0.1 M NaCl fractions. Whereas these enzyme fractions have a low synergistic effect on the activity of elastoproteinase when alkali treated elastin is used as substrate they greatly enhance the activity of elastoproteinase on acid-treated elastin (at both pH 8.7 and 7.2) although here too their own elastolytic activity is very small (lysis of lipid like material is discussed in the next sections).

2.3 Analytical methods

The elastolytic activity of the enzymes and their synergistic effect on the activity of elastoproteinase was determined by methods routinely used in this laboratory (Loeven 1960, 1963a). The methods used for the determination of lipolytic activity on the elastin samples will be described in the relevant sections of the experimental part of this paper. Since the esterolytic activities of the enzymes on other substrates were determined by standard methods, only a reference to the original description of the methods will be given in the corresponding table.

3 EXPERIMENTAL PART

3.1 Esterolytic activities of various enzyme fractions with respect to various different substrates (excluding elastin)

The esterolytic activity of the enzyme fractions isolated from crude AFI and AES samples by the column chromatographic technique described in Section 2.2. (see Fig. 1) was tested on a series of substrates and compared to the activity of pancreatic lipase [a])

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[a]) A commercial lipase preparation from Merck A. G. was used because this preparation is devoid of any protein band in the paper electropherogram except for very pronounced immobile protein material on the line of application. Several other lipase preparations investigated were found to be contaminated with either elastoproteinase or elastomucase or with both elastase component (e.g. lipase from the Nutritional Biochemicals Corp. and from Calbiochem.)

there did not exist only little difference between the action of these enzymes with respect to the release of "lipids" but that treatment of elastin with the elastomucase Em-S (according to the Hungarian workers and the Leeds research group elastolipo-proteinase) gave the lowest values for the dichromate consumption at both pH values investigated (8.7 and 7.2) although this enzyme had nearly completely released the carbohydrate moiety present in alkali treated elastin when only 8–10% of the substrate was solubilized.

Since an enzyme fraction has now been isolated with lipolytic activity and with activity on elastin namely the 0.1 M NaCl fractions, the experiments on the dichromate consumption of chloroform-methanol extracts of elastolyzates were repeated on a smaller scale. Table 4 gives the results of this investigation from which it must be concluded that treatment of elastin with the 0.1 M NaCl fraction indeed results in a much higher dichromate consumption. With alkali-treated elastin as substrate the consumption is about twice and with acid-treated elastin three times higher than when elastin is incubated with the other components of the elastase complex. Here too, Em-S gives the lowest value for the dichromate consumption.

3.3. Lipid solubility and "lipid" content of elastolyzates obtained from acid-treated elastin and calf aorta

Although it has been demonstrated in the preceding section (3.2) that crude elastase preparation indeed contain an enzyme fraction that releases higher amounts of unsaturated lipid-like material from elastin than other components of the elastase complex, the possibility still exists that most of the "lipid" material present in elastin has a saturated character. If this is true the Bragdon method gives only partial information about the content of lipid material. This means that the time-consuming gravimetric method of Sperry (1955) must be used, as done by Banga and Baló (1962) in their investigation into the mode of action of elastoproteinase and elastomucoproteinase (probable identical with our Em-S).

The lipid solubility of the elastolyzates of calf aorta powder lysed out by the various enzymes of the elastase complex was studied as followed.

The enzymes were assayed for activity in 10 ml total volume (Borate buffer of pH 7.2) by the gravimetric method, using a

3.2 Release of lipid-like material from elastin samples determined by the Bragdon method

In a previous study (Loeven 1965b) the release of unsaturated lipid like material from alkali treated and acid treated elastin during the incubation with the components of the elastase complex was measured by determining the dichromate consumption of the elastolyzates according to Bragdon (1951). The elastolyzates were lyophilized and extracted with a mixture of chloroform and dry methanol (3 : 1). In this extract the consumption of dichromate was measured and expressed as mg dichromate reduced by one mg of the original amount of dissolved material. It appeared that

Table 4

Release of lipid-like material from 400 mg elastin after incubation at pH 7.– with various enzymes of the elastase complex. The release is measured in chloroform methanol extracts of the elastolyzates by the method of Bragdon (1951) and expressed as mg dichromate consumed per mg dissolved substrate

		alkali treated elastin		acid-treated elastin	
enzyme	enzyme conc in mg	mg dissolved substrate	dichromate consumption in mg/mg	mg dissolved substrate	dichromate consumption in mg/mg
elastoproteinase	0.1	1 4	0.0	14.1	5.8
Em I	0.2	11.7	7.1	11.4	4.7
Em-S	0.5	9.8	4.7	13.7	3.6
2nd fraction	0.5	8.9	0.5	1[illegible]0	0.1
0.1 M NaCl fraction from AES (prep. I)	1.0 2.0	[illegible]1 4.4	13.6 0.3	7 5.3	18.1 1[illegible]0
0.1 M NaCl fraction from AES (prep. II)	1.0 2.0	4.4 6.4	1[illegible]3 10.5	— 4.7	— 16.9
0.1 M NaCl fraction from AEI	0.5 1.0	5.6 10.6	10.6 8.8	4.4 8[illegible]	16.6 13.9

there did not exist only little difference between the action of these enzymes with respect to the release of "lipids but that treatment of elastin with the elastomucase Em-S (according to the Hungarian workers and the Leeds research group elastolipoproteinase) gave the lowest values for the dichromate consumption at both pH values investigated (8 7 and .2) although this enzyme had nearly completely released the carbohydrate moiety present in alkali-treated elastin when only 8–10 % of the substrate was solubilized

Since an enzyme fraction has now been isolated with lipolytic activity and with activity on elastin, namely the 0 1 M NaCl fractions, the experiments on the dichromate consumption of chloroform-methanol extracts of elastolyzates were repeated on a smaller scale Table 4 gives the results of this investigation from which it must be concluded that treatment of elastin with the 0.1 M NaCl fraction indeed results in a much higher dichromate consumption. With alkali-treated elastin as substrate the consumption is about twice and with acid treated elastin three times higher than when elastin is incubated with the other components of the elastase complex. Here too Em-S gives the lowest value for the dichromate consumption.

3.3. LIPID SOLUBILITY AND 'LIPID' CONTENT OF ELASTOLYZATES OBTAINED FROM ACID-TREATED ELASTIN AND CALF AORTA

Although it has been demonstrated in the preceding section (3.2) that crude elastase preparation indeed contain an enzyme fraction that releases higher amounts of unsaturated lipid like material from elastin than other components of the elastase complex the possibility still exists that most of the 'lipid material present in elastin has a saturated character If this is true the Bragdon method gives only partial information about the content of lipid material. This means that the time-consuming gravimetric method of SPERRY (1955) must be used, as done by BANGA and BALÓ (1962) in their investigation into the mode of action of elastoproteinase and elastomucoproteinase (probable identical with our Em-S).

The lipid solubility of the elastolyzates of calf aorta powder lysed out by the various enzymes of the elastase complex was studied as followed

The enzymes were assayed for activity in 10 ml total volume (Borate buffer of pH 7.2) by the gravimetric method, using a

3 2 Release of lipid-like material from elastin samples determined by the Bragdon method

In a previous study (Loeven 1965b) the release of unsaturated lipid like material from alkali treated and acid treated elastin during the incubation with the components of the elastase complex was measured by determining the dichromate consumption of the elastolyzates according to Bragdon (1951) The elastolyzates were lyophilized and extracted with a mixture of chloroform and dry methanol (3 1) In this extract the consumption of dichromate was measured and expressed as mg dichromate reduced by one mg of the original amount of dissolved material It appeared that

Table 4

Release of lipid-like material from 400 mg elastin after incubation at pH – with various enzymes of the elastase complex The release is measured in chloroform methanol extracts of the elastolyzates by the method of Bragdon *(1951) and expressed as mg dichromate consumed per mg dissolved substrate*

enzyme	enzyme conc in mg	alkali treated elastin: mg dissolved substrate	alkali treated elastin: dichromate consumption in mg/mg	acid treated elastin: mg dissolved substrate	acid treated elastin: dichromate consumption in mg/mg
elastoproteinase	0 1	1 4	0 0	14 1	5.8
Em I	0.2	11 7	7 1	11 4	
Em-S	0 5	9 8	4 7	13.7	
2nd fraction	0,5	8 9	0.5	1_0	
0 1 M NaCl fraction from AES (prep. I)	1 0 _0	[illegible] 4 1		_7 5.3	[illegible]
0 1 M NaCl fraction from AES (prep. II)	1 0 0	4 4 6 4		— 4 7	
0 1 M NaCl fraction from AFI	0.5 1 0	5 0 19 0		4 4 8.2	[illegible] [illegible]

there did not exist only little difference between the action of these enzymes with respect to the release of "lipids" but that treatment of elastin with the elastomucase Em-8 (according to the Hungarian workers and the Leeds research group elastolipoproteinase) gave the lowest values for the dichromate consumption at both pH values investigated (8 7 and 7.2) although this enzyme had nearly completely released the carbohydrate moiety present in alkali-treated elastin when only 8–10% of the substrate was solubilized.

Since an enzyme fraction has now been isolated with lipolytic activity and with activity on elastin namely the 0 1 M NaCl fraction, the experiments on the dichromate consumption of chloroform methanol extracts of elastolyzates were repeated on a smaller scale. Table 4 gives the results of this investigation from which it must be concluded that treatment of elastin with the 0.1 M NaCl fraction indeed results in a much higher dichromate consumption. With alkali-treated elastin as substrate the consumption is about twice and with acid treated elastin three times higher than when elastin is incubated with the other components of the elastase complex. Here too Em-8 gives the lowest value for the dichromate consumption.

3.3. Lipid solubility and "lipid" content of elastolyzates obtained from acid-treated elastin and calf aorta

Although it has been demonstrated in the preceding section (3 2) that crude elastase preparation indeed contain an enzyme fraction that releases higher amounts of unsaturated lipid-like material from elastin than other components of the elastase complex the possibility still exists that most of the "lipid" material present in elastin has a saturated character If this is true the Bragdon method gives only partial information about the content of lipid material. This means that the time-consuming gravimetric method of Sperry (1955) must be used as done by Banga and Baló (196*) in their investigation into the mode of action of elastoproteinase and elastomucoproteinase (probable identical with our Em-8).

The lipid solubility of the elastolyzates of calf aorta powder lysed out by the various enzymes of the elastase complex was studied as followed

The enzymes were assayed for activity in 10 ml total volume (Borate buffer of pH 7 2) by the gravimetric method, using a

substrate concentration of 200 mg aorta powder per 10 ml. The elastolyzates were lyophilized and extracted with a mixture of chloroform methanol (2 1) The total amount of material present in this lipid solvent was determined by the Sperry method without using the purification steps The results presented in Table 5 (3rd and 4th column) show that all the elastolyzates have a high lipid solubility Independent of the kind of enzyme used the percentage of total elastolyzate extractable with the chloroform methanol mixture vary between very narrow limits When however the purification method of Sperry was used afterwards, great differences were found in the quantities of the lipid-soluble material depending on whether elastoproteinase or the elastomucases or the 0 1 M NaCl fractions were applied

TABLE 5

Lipid solubility and lipid content of elastolyzates obtained from 00 mg calf aorta by various enzymes of the elastase complex at pH 7.-

enzyme	elasto lyzate (in mg dry weight)	chloroform-methanol extract: before Sperry purific. mg dry weight	of total elasto lyzate	after Sperry purific. mg dry weight	of total elasto-lyzate
elastoproteinase (0 -5 mg)	50 3	21 0	37	2.3	4
Em I (0 5 mg)	40 7	13 1	3	3 9	10
Em S (1 mg)	9 1	10 1	35	8 5	29
0 05 M NaCl fr / AES (mg)	50 7	15 4	30	4 1	8
0 1 M NaCl fr / AES (2 mg)	33 0	1 .3	30	6 4	-5
0 1 M NaCl fr / AFI (mg)	36.5	1* 3	34	7 7	21

Em-S and the 0 1 M NaCl fractions lyse out 5 to 7 times more lipid-soluble material than elastoproteinase while Fm I and the 0 05 M NaCl fraction (partially denatured Fm-S) gives a much lower percentage of extracted material These values are correspond ent closely to those given by BANGA and BALÓ (106*) The only discrepancy lies in the fact that whereas these authors reported

TABLE 6

Lipid solubility and "lipid" content of elastolyzates obtained from 400 mg acid-treated elastin by various enzymes of the elastase complex at pH 7.2.

enzyme	elastolyzate (in mg dry weight)	chloroform-methanol extract: total dry weight in mg	dried extract in % of total elastolyzate	protein content in % of dried extract	"lipid" content in % of dried extract
elastoproteinase (0.25 mg)	46.9	20.6	44	97	3
Em-I (0.5 mg)	20.6	12.3	60	75	25
Em-8 (1 mg)	5.7	5.8	100	42	58
0.05 M NaCl fr./ AE8 (2 mg)	16.1	8.9	56	93	7
0.1 M NaCl fr./ AE8 (2 mg)	7.4	7.2	97	35	65
0.1 M NaCl fr./ AEI (2 mg)	10.4	9.2	88	58	42

that about 70 per cent of the elastolyzates of calf aorta dissolved in the lipid solvent, we found about half of this percentage. A possible explanation may be that they concentrated the elastolyzates to 10 per cent of their original volume before the extraction procedure and we completely dried the elastolyzates (in this way already freeing the lipid soluble material from loosely-bound protein).

Table 6 gives the results of an analogous experiment done with 400 mg acid-treated elastin as substrate. Since we agree with the Hungarian workers that it is not only very difficult to remove all the lipid-soluble protein from the lipid fraction by Sperry's method of purification but furthermore a considerable loss of the "lipid" has been observed in some cases, the purification step was entirely omitted.

Thus, we determined in the chloroform-methanol extract the total amount of extracted elastolyzates (gravimetrically) and the amount of lipid-soluble protein (LOWRY method, 1951). The Table shows that whereas in the case of elastoproteinase Em I, and the 0.05 M NaCl fraction 44 to 60 per cent of the total elastolyzates

substrate concentration of 200 mg aorta powder per 10 ml The elastolyzates were lyophilized and extracted with a mixture of chloroform methanol (2 1) The total amount of material present in this lipid solvent was determined by the Sperry method without using the purification steps The results presented in Table 5 (3rd and 4th column) show that all the elastolyzates have a high lipid solubility Independent of the kind of enzyme used the percentage of total elastolyzate extractable with the chloroform methanol mixture vary between very narrow limits. When however the purification method of Sperry was used afterwards, great differences were found in the quantities of the lipid-soluble material depending on whether elastoproteinase or the elastomucases or the 0 1 M NaCl fractions were applied

TABLE 5

Lipid sol ibil ty and lipid' content of elastolyzates obtained from 00 mg calf aorta by various enzymes of the elastase complex at pH 7 2.

enzyme	elasto lyzate (in mg dry weight)	chloroform methanol extract			
		before Sperry purific.		after Sperry purific	
		mg dry weight	of total elasto lyzate	mg dry weight	of total elasto-lyzate
elastoproteinase (0 5 mg)	56.3	21 0	37	.3	4
Em I (0 5 mg)	40 7	13 1	32	3 9	10
Em-S (1 mg)	29 1	10 1	35	8.5	29
0 05 M NaCl fr / AES (- mg)	50 7	15 4	30	4 1	8
0 1 M NaCl fr / AES (mg)	33 6	12.-	30	8.4	25
0 1 M NaCl fr / AEI (mg)	30 5	1 3	34	7 7	21

Em-S and the 0 1 M NaCl fractions lyse out 5 to 7 times more lipid-soluble material than elastoproteinase while Em I and the 0 05 M NaCl fraction (partially denatured Em-S) gives a much lower percentage of extracted material These values are correspond ent closely to those given by BANGA and BALÓ (1962) The only discrepancy lies in the fact that whereas these authors reported

TABLE 6

Lipid solubility and "lipid" content of elastolyzates obtained from 400 mg acid-treated elastin by various enzymes of the elastase complex at pH 7.2.

enzyme	elastolyzate (in mg dry weight)	chloroform-methanol extract: total dry weight in mg	chloroform-methanol extract: dried extract in % of total elastolyzate	chloroform-methanol extract: protein content in % of dried extract	chloroform-methanol extract: "lipid content in % of dried extract
elastoproteinase (0.25 mg)	46.9	20.6	44	97	3
Em-I (0.5 mg)	20.6	12.2	60	75	25
Em-8 (1 mg)	5.7	5.8	100	42	58
0.05 M NaCl fr./ AE3 (2 mg)	16.1	9.0	56	93	7
0.1 M NaCl fr./ AE3 (2 mg)	7.4	7.2	97	35	65
0.1 M NaCl fr./ AEI (2 mg)	10.4	9.2	88	58	42

that about 70 per cent of the elastolyzates of calf aorta dissolved in the lipid solvent, we found about half of this percentage. A possible explanation may be that they concentrated the elastolyzates to 10 per cent of their original volume before the extraction procedure and we completely dried the elastolyzates (in this way already freeing the lipid soluble material from loosely-bound protein).

Table 6 gives the results of an analogous experiment done with 400 mg acid treated elastin as substrate. Since we agree with the Hungarian workers that it is not only very difficult to remove all the lipid-soluble protein from the lipid fraction by Sperry's method of purification but furthermore a considerable loss of the "lipid" has been observed in some cases, the purification step was entirely omitted.

Thus, we determined in the chloroform-methanol extract the total amount of extracted elastolyzates (gravimetrically) and the amount of lipid-soluble protein (LOWRY method, 1951). The Table shows that whereas in the case of elastoproteinase Em I, and the 0.05 M NaCl fraction 44 to 60 per cent of the total elastolyzates

could be taken up in the lipid-solvent nearly 100% was lipid soluble when elastin was incubated with either Em-S or the 0.1 M NaCl fraction from crude AES samples. The fact that the elastin protein is also highly soluble in a chloroform methanol mixture is clearly demonstrated by column 5 of this Table. Whereas with the Bragdon method as discussed in the preceding section, only the 0.1 M NaCl fractions were found to have a higher activity in liberating lipid like material from elastin than elastoproteinase and the elastomucases the results of the experiments summarized in Tables 5 and 6 argue in favour of the opinion that elastomucase Em-S too is able to remove lipid like material from purified elastin and aorta powder.

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Already LANSING *and co-workers* (1952, 1953) showed that during elastolysis lipid like material was released from elastic fibres in the form of droplets stainable with sudan dyes (as seen under the microscope). This lipid fraction makes up a mere 0.5% of the elastin protein (LABELLA 1957) and has a sphingomyelin character (LANSING 1954). Later the heterogeneity of the lipid-soluble material was studied.

Whereas LABELLA (1961, 1962) showed that the yellow pigment in elastin powder consists of non-extractable plasmalogen, unsaturated fatty acid and polyphenolic substances, SINEX and FARIS (1962) stated that the source of both the pigmentation and the fluorescence observed in purified elastin is autoxidized lipid. KÄRKELÄ and KULONEN (1957) also suggested that more than one non porphyrin pigment is present in elastin, some of them being fluorescent and many of them showing properties indicative of the presence of oxidized lipid. A number of ether-soluble substances consisting of fluorescent compounds of lipid character have been isolated from elastolyzates by LOOMEYER (1961).

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associated with the elastic fibres and probably form an essential part of the elastic structure was demonstrated in various ways. WALFORD *et al.* (1961) showed that during elastolysis of ligamentum nuchae the fluorescent material remained bound to the elastase-resistant, soluble elastin protein moiety. Further LABELLA (1961 1962) found that a trichloro-acetic acid-insoluble fraction is associated with the yellow pigment, is Schiff positive and remains non-dialyzable throughout, as also shown by WALFORD *and co-workers*. When extracting human aorta powder with chloroform methanol and afterwards hydrolyzing the residue with hydrochloric acid, BÖTTCHER *et al* (1959) still found a few mg of lipids in a petroleum ether extract of this HCl hydrolyzate consisting mainly of fatty acids.

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incubation of elastin with Em I and Em-S has been completely confirmed by extensive experiments (LOEVEN 1965b)

The controversial point that the authors mentioned above ascribed a lipolytic character to elastomucoproteinase or E_1 whereas both Em I and Em-S are completely devoid of any activity on serum and synthetic "lipid" substrates may be only apparent if the assumption is made that the enzyme preparations used in their experiments were contaminated with the 0 1 M NaCl fraction that indeed shows lipolysis towards a series of substrates and at the same time has synergistic effect on the elastolytic activity of elastoproteinase and releases a *lipid-soluble moiety from calf aorta* and acid treated elastin

Two *facts may support this assumption* In the first place HALL (1961) and SAXL (1957 1961 1962) used the rather crude AES-enzyme preparation from which the 0 1 M NaCl fraction can be isolated (see the Figs 1 and 2) To what extent the elastomucoproteinase preparation of BANGA and BALÓ (1962) was contaminated with this 0 1 M NaCl fraction is unknown since they separated the components of the elastase complex by means of a modified starch gel method of Smithies Therefore there is a real possibility that the separation of elastomucoproteinase and the 0 1 M NaCl fraction was incomplete (the paper electropherogram of the purified elastomucoproteinase given in their paper only shows the cathodic side of the paper strips)

Secondly the results of the experiments of HALL (196) concerning *the possible lipoprotein lipase activity* of crude AES in which Tween 20 was used as substrate (without or in the presence of serum *lipoprotein fractions, heparin and* NaCl) *are so closely* related to the results of the experiments with the 0 1 M NaCl fraction from AES (see Table 3) *that it seems very likely that* this enzyme fraction was also present in his crude preparation

Thus, two enzyme fractions can be isolated from crude *elastase* preparations with such an activity on elastic tissue that in both cases lipid-soluble moieties are released However the *lysis of this* material in any case occurs in different ways.

HALL (1961) in discussing the experiments of SAXL (*1961 1962*) and YU and BLUMENTHAL (1958) suggested that the polysaccharides are attached to elastin through the lipoprotein components (= *the* mucolipoprotein complex of BANGA and BALÓ 1962) and that the

release of these polysaccharides by the mucolytic elastase components may be due to the degradation of the lipid moiety of elastin with which the polysaccharides are associated. From the results of the investigations described in this and previous papers it seems more attractive to draw the conclusion that the reversal is true. The mucolytic elastase components (Em-S and Em I) although both of them act by virtue of a different unknown mechanism primarily attack the polysaccharide part of this mucolipoprotein complex in such a way that the lysed moiety is lipid-soluble, whereas the lipolytically active 0.1 M NaCl fraction degrades the lipid part of this complex, also resulting in the liberation of lipid-soluble material. This assumption may explain why in the latter case a much higher dichromate consumption per mg elastolyzate was measured (see Table 4) and why HALL (1964) mentioned that during the fission of ester linkages, unesterified fatty acids are released into the elastolyzates [4])

Another argument for the hypothesis that the reaction mechanism of elastolipoproteinase differs from that of the elastomucases is found from an experiment shown in Fig. 3. In this experiment, the enhancement of the activity of elastoproteinase on acid treated elastin was measured by adding to the reaction mixture the elastomucase Em I or Em-S and the elastolipoproteinase. If these enzymes act in the same way we must expect a summation of their synergistic effects, since this effect is rather proportional to the enzyme concentration and at low concentrations of elastoproteinase only slightly dependent of the concentration of this enzyme. However, we found that whereas at 0.05 mg elastoproteinase 0.25 mg Em I has an activation activity of 1.4% and

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[4]) The presence of fatty acids in elastin has already been demonstrated by LOOMEYER (1961), who isolated from structurally-bound lipopeptide units in elastin a possible ketonic acid with a chain length of about 12 carbon atoms.

When the present author measured the fatty acid concentration in various kinds of elastin samples by means of the Dole-Meinertz-titration method (1960) after the hydrolysis of elastin with methanolic 1 N KOH or 5% H_2SO_4, the following fatty acid content were calculated (expressed in μ equiv. fatty acid/1 gr. elastin powder):

crude ligamentum nuchae 6.4–24.1

acid-treated elastin 0.9– 2.4;

alkali-treated elastin 0.2– 1.0.

2 mg elastolipoproteinase of 25% the elastolysis was increased by 190% when both enzymes were present in the reaction mixture. The same effect was observed when Em-S and the elastolipoproteinase were added together activation activity of Em-S was 107% at 0.05 mg elastoproteinase that of elastolipoproteinase 25% but the synergistic effect of Em-S+elastolipoproteinase was 204%

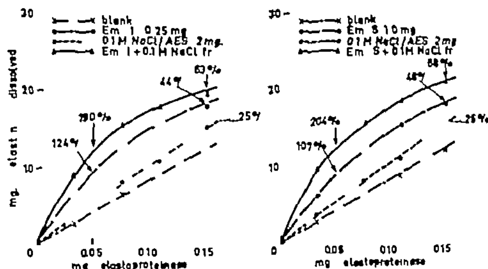


Fig 3

The activity of elastolipoproteinase (present in the 0.1 M NaCl fraction from AES) on acid treated elastin at pH 7 when the reaction mixture *also contains elastoproteinase and the elastomucase Em I or Em-S*

So the following recommendation with regard to the nomenclature of these enzyme fractions may be presented that the name Ei or elastomucase not be changed in elastolipoproteinase as suggested by HALL (1964) but that this name be reserved for the β-globulin like enzyme fraction present in the 0.1 M NaCl fraction prepared from crude AEI and AES

The data given in section 3.1 have clearly demonstrated that the lipolytic activity is not due to a contamination of the elastolipoproteinase sample with pancreatic lipase or ali-esterases. This conclusion is supported by the following observations

a. even a concentration of 10^{-2} M eserine in the system does not inhibit the enzyme activity

b. while taurocholate is an activator for lipase this reagent is nearly without effect on the activity of the 0.1 M NaCl fraction when Tween 20 is used as a substrate. The bile salt Na-deoxycholate enhances the enzyme activity on Tween 20 at pH 7.2, but reacts as an inhibitor with serum as substrate.

c. MATTSON and BECK (1955) reported that 1 M NaCl (or NH_4Cl or $(NH_4)_2SO_4$) enhanced the activity of lipase, whereas in our case 1 M NaCl inhibits the activity of the 0.1 M NaCl fraction, especially on serum.

Further the thermolability shows that the presence of pancreatic phospholipase A[5] can also be excluded. When an enzyme solution was heated for 5 min at 75° C, no activity was observed on human serum at pH 8.7 and a greatly reduced activity was found at pH 7.2 (a residual activity of about 10–15%). While the presence of bile salts is required in the reaction mixture for a reasonable activity of phospholipase A (especially sodium-deoxycholate) elastolipoproteinase can attack ovolecithin to a certain extent in the absence of deoxycholate and is slightly inhibited by this bile salt. Phospholipase A and purified lipase have no activity against acid treated elastin and do not enhance the activity of elastoproteinase. Already ADAMS and BAYLISS (1962) reported that elastase has proteolytic, mucolytic, and lipolytic activity against elastic tissue (so a crude enzyme preparation was probably used) but that these actions were not due to contaminations with lipase or phospholipase A, as could be demonstrated by inhibitor studies.

The following observations are in favour of a close relationship between this elastolipoproteinase and the post-heparin lipoprotein lipase (L.P.L.)

a. L.P.L. is present in the β-lipoprotein fraction of post-heparin plasma (e.g. HOOD *et al.*, 1962). The elastolipoproteinase also has a β-globulin character[6]).

b. As L.P.L. elastolipoproteinase is very labile during dialysis,

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[5]) A phospholipase A preparation was isolated from Organon pancreas powder according to the method of MAGEE *et al.* (1962). Fraction D was used.

[6]) As will shortly be published, the α_2-globulin-like protein present in the 0.1 M NaCl fraction isolated from crude AES is devoid of any lipolytic activity against elastin and behaves in a totally different way against lipid-substrates.

during the purification step by means of column chromatography (see e.g. HALL, 1964) and against heat.

c. Potent L.P.L. inhibitors are 1 M NaCl, protamine sulfate, heparin and bile salts (see e.g. KORN, 1959; ROBINSON, 1963), although the inhibitory effect sometimes depends on the kind of substrate used and the concentration of the reagent (FREDRICKSON et al., 1963; KESSLER et al., 1963; SHORE and SHORE, 1961). Very similar data were collected concerning the inhibition of elastolipoproteinase by these reagents, especially with human serum as substrate (see Table 3). Here too, eserine does not behave as an inhibitory agent.

d. B-Naphthyllaurate is not a substrate for either L.P.L. (BROWN et al., 1953, 1954) or elastolipoproteinase (see Table 2).

e. Both L.P.L. and elastolipoproteinase need the presence of a fatty acid acceptor for suitable enzyme activity (e.g. Ca-salt or albumin). When Tween 20 was used as substrate the activity of elastolipoproteinase was enhanced by more than 100% if 5% bovine albumin was present in the reaction mixture (especially at pH 7.2). The addition of bovine albumin to a Tween 20 solution had almost no effect on the activity of lipase.

f. KERN et al. (1961) reported that some stabilizers present in human albumin preparations (e.g. Na-caprylate) have an inhibitory effect on the activity of L.P.L., and ANGERVALL and HOOD (1957), HOOD and ANGERVALL (1957) and HOLLET and MENG (1957) showed that normal human plasma inhibits L.P.L. activity. On chromatography, inhibitory fractions were isolated by HOOD et al. (1962). To investigate to what degree albumin inhibits the activity of elastolipoproteinase, the esterolytic activity on human serum was measured without and after the addition of an excess of 5% albumin. Whereas bovine albumin did not inhibit the enzyme activity, a 5% solution of human albumin (Central Laboratory of the Netherlands Red Cross Organization) had an inhibitory effect of 50–80% depending on the pH of the reaction mixture. A crude albumin preparation isolated from pooled human plasma by means of a column chromatography technique (LOEVEN, 1962) showed a much weaker effect: 15–20% inhibition. Thus it seems that human blood contains an inhibitor probably present in the albumin fraction.

To summarize, the conclusion may be drawn that all these

observations, although they still do not permit any deductions about the total indentity of elastolipoproteinase and L.P.L., are in any case arguments for a close relationship between these two enzymes. In this connection we may also remember the experiments of SAXL (1957 1963) on the *in vitro* clearing reaction in connective tissue of AES (together with plasma β-globulin and limited concentration of sulphated polysaccharides) her studies *in vivo* of the influence of the injection of AES on the lipid metabolism in the aorta of chicken receiving a cholesterol-enriched diet (e.g the synthesis of elastin lipoprotein) and the experiments of LOEVEN (1964) on the therapeutical effect of elastolipoproteinase injected into cholesterol-fed rabbits (e.g resulting in a transformation of plasma β- into α-lipoprotein)

SUMMARY

The release of lipid-like material was studied by incubating acid-treated elastin and calf aorta powder with various enzymes of the elastase complex isolated from commercial pancreas powder by means of a column chromatographic technique.

Although the mucolytic components of the elastase complex show no esterolytic activity against synthetic "lipid" -substrates and human serum, especially the elastomucase Em-8 releases large amount of lipid-soluble material from elastin

Some characteristics of another pancreatic enzyme, which also has lipolytic activity against elastin but at the same time shows esterolytic activity against other substrates, is discussed. The hypothesis is put forward that although both this enzyme and the elastomucase attack the so-called elastin mucolipoprotein complex their enzymic mechanism differs such that elastomucase primarily liberates the carbohydrate moiety of this complex and the other enzyme (called elastolipoproteinase) the lipid moiety

Lastly some remarks are made concerning the possibility that the lipolytic elastase component and the post heparin lipoprotein lipase are closely related to each other

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f. KERN *et al.* (1961) reported that some stabilizers present in human albumin preparations (e.g. Na-caprylate) have an inhibitory effect on the activity of L.P.L. and ANGERVALL and HOOD (1957), HOOD and ANGERVALL (1957) and HOLLETT and MENG (1957) showed that normal human plasma inhibits L.P.L. activity. On chromatography inhibitory fractions were isolated by HOOD *et al.* (1962). To investigate to what degree albumin inhibits the activity of elastolipoproteinase, the esterolytic activity on human serum was measured without and after the addition of an excess of 5% albumin. Whereas bovine albumin did not inhibit the enzyme activity, a 5% solution of human albumin (Central Laboratory of the Netherlands Red Cross Organization) had an inhibitory effect of 50–80% depending on the pH of the reaction mixture. A crude albumin preparation isolated from pooled human plasma by means of a column chromatography technique (LOEVEN 1962) showed a much weaker effect: 15–20% inhibition. Thus it seems that human blood contains an inhibitor probably present in the albumin fraction.

To summarize, the conclusion may be drawn that all these

observations, although they still do not permit any deductions about the total indentity of elastolipoproteinase and L.P.L. are in any case arguments for a close relationship between these two enzymes. In this connection we may also remember the experiments of BAXL (1957 1962) on the *in vitro* clearing reaction in connective tissue of AES (together with plasma β-globulin and limited concentration of sulphated polysaccharides) her studies *in vivo* of the influence of the injection of AES on the lipid metabolism in the aorta of chicken receiving a cholesterol-enriched diet (e.g the synthesis of elastin lipoprotein) and the experiments of LOEVEN (1964) on the therapeutical effect of elastolipoproteinase injected into cholesterol fed rabbits (e.g resulting in a transformation of plasma β- into α lipoprotein)

SUMMARY

The release of lipid-like material was studied by incubating acid-treated elastin and calf aorta powder with various enzymes f the elastase complex isolated from commercial pancreas powder by means of a column chromatographic technique.

Although the mucolytic components of the elastase complex show no esterolytic activity against synthetic "lipid"-substrates and human serum, especially the elastomucase Em-B releases large amount f lipid-soluble material from elastin.

Some characteristics of another pancreatic enzyme which also has lipolytic activity against elastin but at the same time show esterolytic activity against other substrates, is discussed. The hypothesis is put forward that although both this enzyme and the elastomucase attack the so-called elastin mucolipoprotein complex, their enzymic mechanism differs such that elastomucase primarily liberates the carbohydrat moiety of this complex and the other enzyme (called elastolipoproteinase) the lipid moiety

Lastly some remarks are made concerning the possibility that the lipolytic elastase component and the post-heparin lipoprotein lipase are closely related to each other

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A. Berkenbosch *The amperometric oxygen determination*

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Equations are derived for the sensitivity the time course of response and the flow dependence of the membrane-covered O_2 electrode These properties are determined by the cathode area and membrane characteristics such as its diffusion coefficient solubility coefficient and thickness.

The ideal electrode with large sensitivity short response time and negligible flow dependence is impossible to achieve while maintaining the Clark construction.

In order to realize particular electrode properties one has to choose the area of the platinum and the nature and thickness of the membrane to be used Some compromise solutions are given in the light of the membrane characteristics we have determined.

The use of high density low pressure polythene as a membrane is to be preferred to that of the polypropylene in common use since the sensitivity of the electrode can be approximately five times as large while the same flow dependence and response time are maintained (enlarging of the platinum area)

The use of low density high pressure polythene is recommended in order to obtain a very short response time. The diffusion coefficient of this material is greater than that of Teflon T.F.E. so that the response time is shorter

The time in which 95% of the steady-state value is reached with a membrane of a thickness of approximately 6 μ is found to be 0.15 sec.

A J van Eick *A change in the response of the mouse in the "hot-plate" analgesia-test owing to a central action of atropine and related compounds*

Medical Biological Laboratory of the National Defense Research Organization TNO Rijswijk Z.H

In the hot-plate analgesia test according to Eddy a lengthening of the latency period between placing a mouse on the plate and the moment of its licking a foot is considered to be a sign of an

analgesic effect of a drug During the study of the effects of atropine sulphate with this method an unexpected lengthening of the latency period was found However close observation of the behaviour of these animals revealed evident signs of discomfort but instead of showing the usual reaction of licking a foot, the mice respond by lifting a hind foot somewhat sideways immediately followed by short and very quick trembling of this leg and foot Henceforth this phenomenon will be called 'leg trembling' A series of experiments with atropine sulphate was performed in which the leg trembling was used as a criterion The latency periods now obtained were not different from those of saline-injected mice with foot licking as a criterion Obviously atropine has no analgesic properties but it only changes the response of the mouse to the heat stimulus. This change in behavioural response was also observed after the injection of scopolamine — HCl benactizine and a few other atropine-like drugs

In order to determine whether this effect of the drugs is due to central or peripheral actions the effectiveness of quaternary salts of atropine and scopolamine was also tested In addition both the tertiary and quaternary salts of the same substances were tested for their mydriatic effect It was found that after the injection of the quaternary salts the reaction to the heat stimulus was normal (foot-licking) only after comparatively high doses of scopolamine methylnitrate there was a change in the leg trembling reaction Both the tertiary and the quaternary salts caused mydriasis, but the quaternary ones were the more active

In a few experiments with LSD 25 morphine histamine eserine and a few other substances not related to atropine, the anomalous behaviour was not found

The conclusion appears to be justified that atropine-like drugs can cause a change in the reaction from foot-licking into leg trembling and that this effect is of central origin If this is so the hot-plate analgesia test can be used with advantage for the study of the central activity of atropine and related compounds

It is known that the psychotropic effects of JB-329 (Ditran) can be antagonized by THA (tetrahydroaminacrine) Preliminary experiments indicate that THA is also able to antagonize the change in behavioural reaction on the hot-plate caused by atropine-like drugs.

P J Gaillard *Thyrocalcitonin and bone in vitro*
Department of Cell Biology and Histology University of Leiden

Accepting that bone is the principal site of action of Thyrocalcitonin (TC), its mechanism of action is still unknown. Therefore it was thought to be of interest to study the effect(s) of TC on explanted mouse radius rudiments, which in previous experiments have shown to be very sensitive and useful target organs for parathyroid hormone (PTH) (GAILLARD 1961) Considering that TC and PTH may be antagonists, two types of experiments were performed, namely with TC alone and with simultaneous administration of TC and PTH

It is concluded that TC (0 001–0.0005 MRC-units per radius and per ml of culture medium) is able to influence the histological development of the embryonic radii. In the course of a 48-hour cultivation period typical highly basophylic osteoblasts increased in number rather considerably suggesting that TC caused the balance between bone formation and bone resorption to shift into the direction of bone formation. From the histological observations it is suggested that TC caused a depression of bone resorption as well as an increased formation of bone.

As regards the combined administration of PTE and TC it became evident that in the presence of 0 0005 MRC-units of TC, relatively high concentrations of PTE (1 and 0 1 IU/ml) continued to act, but that the action of a lower dose (0 01 IU of PTE/ml) was completely abolished by the simultaneous presence of TC. This finding implies that not only the effects of PTE on bone tissue proper but also those on cartilage and on the connective tissue elements inside the shaft did not express themselves adequately Therefore it is suggested that TC does not act only on bone but on a variety of connective tissues.

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D A de Groot and R Rijken *Rhythmic fluctuations in the ascorbic acid content of kidneys and liver in pseudopregnant immature rats*
Department of Pharmacology University of Leiden

Pseudopregnant recipient rats of the bioassay of PARLOW (1958)

for luteinizing hormone are subject to a decrease in their ovarian ascorbic acid content (LAWTON and SCHWARTZ, 1965) which is probably not caused by an endogenous release of luteinizing hormone since it is also found in *hypophysectomized recipients* (DE GROOT 1965) RINNE and KYTÖMÄKI (1961) have shown that in intact rats a similar fluctuation exists in adrenal ascorbic acid

The present experiments were performed in order to investigate whether spontaneous fluctuations in ascorbic acid also occur in other tissues of the rat Kidney and liver ascorbic acid contents were measured at 10 a.m midday 2 and 4 p.m Kidney ascorbic acid amounts to about 14 mg% it shows a non-significant decrease between 2 and 4 p.m. In the same time interval liver ascorbic acid fell from 18 5 mg% to 16 1 mg% and this was a significant decrease with $p < 0\ 05$

Since ascorbic acid is formed out of glucose (HOROWITZ and KING 1953) blood sugar values were determined in another group of recipients they were bled only once a day so as to avoid stress side reactions As was to be expected in non fasted rats blood sugar levels gradually decreased during the day the lowest levels being found at 4 p.m. The only significant decrease however was found between midday and 2 p.m. These data could suggest a possible connection between an optimal value of blood sugar levels and the maximal ascorbic acid content *in all organs concerned.*

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J W Hekkelman *Alkaline phosphatase and the metabolism of bone tissue*

Laboratory of Cell Biology and Histology University of Leiden

In a study on the mechanism of action of parathyroid hormone (PTH) on bone metabolism it was found that this hormone causes a decrease in the NADP content of the bone cell (HEKKELMAN 1965 HERMANN-ERLEE 1966) More recently (HEKKELMAN 1966) it was

shown that the break-down of NADP in bone tissue occurs almost entirely via a dephosphorylation by an alkaline phosphatase. This enzyme has a very high activity in bone tissue and is located for a large part in membranes, which makes it improbable that the described reaction is its main task.

In connection with this consideration, it is of interest that in purified bone extracts an ATPase activity was found which could not be separated from the phosphatase activity by electrophoresis thermal inactivation, and variation in pH. The phosphatase activity was determined from the rate of formation of NAD from NADP. Moreover a mutual inhibition by the two substrates was found in concentrations of 1×10^{-4} M ATP and 2×10^{-4} M NADP respectively. The inhibition by beryllium ions appeared to run parallel for both activities.

These results lead to a hypothesis in which it is assumed that the alkaline phosphatase from bone tissue is connected with a transport system for phosphate or phosphate and calcium ions. During desintegration of this complex structure the function and possible specificity of the phosphatase are lost resulting in the appearance of a non-specific alkaline phosphatase in serum.

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A. M. van der Poel. *Ethological study of the behaviour of the albino rat in a "passive-avoidance"-test*

Medical Biological Laboratory of the National Defense Research Organization TNO, Rijswijk Z.H.

The experimental design perhaps most commonly used in pharmacological research on memory is the "passive-avoidance"-test. The procedure used in the present study is essentially the same as used by BURES *et al.* (1964). There are at least three sessions. In

the course of a session one rat at a time is allowed to explore the apparatus for 2 min. The apparatus consists of a main bo and a gridbox of equal dimensions. After the second session half the animals are given an electrical pain-stimulus in the gridbox

In this situation rats may perform more than 20 different stereotyped behavioural elements (SBE's) Only four of these SBE's will be described here

1 *crossing* into the other compartment A crossing is considered to be complete when all four feet have passed through the hole connecting mainbox and gridbox. Crossings can of course be made in both directions

2 *head in gridbox* or *head in mainbox* These SBE s are assumed to be incomplete crossings. After the pain-stimulus the frequency of head in gridbox is significantly higher than before

3 *stretching* Rats performing stretching stand still or slowly move forward With the phenomenon in its highest intensity the whole body is stretched. The hind legs are very tense whereas the front part of the animal appears to be much less inhibited. Especially the head may make scanning movements in frequencies also seen in normal exploring animals Rats display stretching when placed in a strange environment and very frequently after the pain-stimulus. In the latter situation the stretched animal always aims at the entrance of the gridbox and may even perform head in gridbox at the same time

4 *walking backwards* This is considered to be pure flight-motivated behaviour for it is only shown after the pain-stimulus and it is always directed away from the entrance of the gridbox. Very frequently it is preceded by stretching

Very likely stretching is an ambivalent movement. Being exploration under inhibition stretching is followed in most cases either by normal walking forward and exploration or walking backwards Moreover walking backwards is very easily released by sudden noises in animals performing stretching This proves that stretched rats are highly flight-motivated.

The more the stimulated rats approach the entrance of the gridbox, the more they display flight-motivated behaviour Therefore these rats avoid the gridbox almost completely It also implies that the avoidance is less passive than might be concluded from the expression passive-avoidance test.

A few experiments have been made with cardiazol. It was found that cardiazol given in convulsant doses 24 hrs. after the pain stimulus induces only a partial retrograde amnesia in about 25–30% of the animals using the above-mentioned SBE's as criteria for retrograde amnesia.

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R. L. Polak *Uptake of acetylcholine by the isolated cerebral cortex of the rat*

Medical Biological Laboratory of the National Defense Research Organisation TNO Lange Kleiweg 139 Rijswijk (Z.H.)

When cerebral cortex slices from rats after pretreatment with the potent cholinesterase inhibitor soman are incubated in a glucose and soman containing Krebs solution under 95% O_2 and 5% CO_2 at 37° C, to which ACh has been added part of this ACh accumulates in the tissue (POLAK and MEEUWS 1966).

In the present experiments it was found that dinitrophenol, amytal and parachloromercuribenzoate inhibit this ACh accumulation whereas ouabaine or omission of KCl from the medium have little effect. The uptake process has a $Q\,10 = 2$.

Accumulation of ACh in the isolated cerebral cortex is also antagonized by hemicholinium and by comparatively high concentrations of choline atropine and the cholinesterase inhibitors eserine and O-ethyl S-diethylaminoethyl ethylphosphonothiolate but not by soman and tabun.

Some similarities between the uptake of ACh by cerebral cortex slices and the active choline transport by the squid axon (HODGKIN and MARTIN 1965) were discussed.

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the course of a session one rat at a time is allowed to explore the apparatus for 2 min The apparatus consists of a main box and a gridbox of equal dimensions After the second session half the animals are given an electrical pain-stimulus in the gridbox.

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Oxygen affinity expressed as half saturation value (p_{50}) 7–40 mm O_2 Bohr-effect, expressed as $\Delta \log p_{50}/\Delta pH$ −1 60 to −1 06 Hill's constant n 4.4−3.5 Na^+ 486−40* meq/l K^+ 10−5 meq/l Ca^{++} 30−9 meq/l Mg^{++} 12−1 meq/l.

The influence of NaCl KCl, $CaCl_2$ and $MgCl_2$ on the oxygen-binding properties of haemocyanin was investigated. NaCl and KCl up to a concentration of 5 molal increase the oxygen affinity the Bohr-effect is unaffected $CaCl_2$ and $MgCl_2$ however both raise the oxygen affinity as well as the Bohr-effect The results with $CaCl_2$ at high ionic strength are in contradiction with those obtained by PICKETT RIGGS and LARIMER (1966) All salts tested increase the value of "n" Probably the influence of NaCl and KCl on n" is small and independent of the pH whilst that of $CaCl_2$ shows a definite pH-optimum

The varying oxygen-binding properties of the blood can only partly be explained by the variation in salt content of the blood. Other salts, metabolic products, or specific differences in haemocyanin may also play a part. Starch gel electrophoresis of the haemocyanin shows the existence of 2 slow and 4 fast components. The slow component when separated on Pevikon" shows an oxygen equilibrium curve that is slightly more S-shaped than the curve of total blood. Probably under physiological conditions the haemocyanin consists of only one component with M.W 820 000 Some of the haemocyanin molecules are split when both Ca^{++} and Mg^{++} concentrations are low and pH is high, whilst the others, representing the slow component on starch gel do not dissociate so easily

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W J Rietveld W E M Tordoir J R B Hagenouw J A Lubbers and Th A C Spoor *Visually evoked responses to blank and to checkerboard patterned flashes*

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J J Schipperheyn *Influence of eye movements on retinal ganglion cell activity in frogs*

Department of Physiology University of Leiden

In jumping a frog is obviously well informed about the location of standing objects In the majority of retinal ganglion cells a sustained response to stationary retinal images is absent The respiratory eye movements serve to sustain this response Employing extra-cellular recordings from tectal endings of optic nerve fibres the responses to eye movement simulating object displacements were studied It was concluded that owing to eye movements all known types of retinal ganglion cells signal the presence of standing objects As a result of adaptation to stereotyped stimulation the responses to standing objects, induced by eye movements, are suppressed in favour of the signals evoked by moving objects

The influence of eye movements on the ganglion cell response to moving objects was also studied Eye movements were found to increase the sensitivity to objects moving at low angular velocities. The feature was especially prominent in on/off type ganglion cells where the response to objects moving at angular velocities under 0 1 °/sec is otherwise absent

G L Spoek *The influence of salts on the binding of oxygen by the haemocyanin of the lobster Homarus gammarus L*

Laboratory of Zoology Division of Animal Physiology University of Leiden

The blood of different specimens of the lobster varies in oxygen affinity the magnitude of the Bohr-effect, the shape of the oxygen equilibrium curve (the value of the interaction coefficient) and the concentration of NaCl KCl $CaCl_2$ and $MgCl_2$ At the physiological pH of 7 6 the following maximum and minimum values were found

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NETHERLANDS SOCIETY FOR ENDOCRINOLOGY

ABSTRACTS OF PAPERS READ UNDER THE AUSPICES OF THE SOCIETY AT THE EIGHTH FEDERATION MEETING OF MEDICAL-BIOLOGICAL SOCIETIES GRONINGEN APRIL 6–7 1967

All types of pretreatment did indeed enhance ovarian reactivity for low doses of LH b) and c) more consistently than a). With a) some recipents lose their pseudopregnancy with b) recipients may be weakened by side-effects of the tranquillizer Larger doses of LH can be ineffective with any of the three pretreatment systems.

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Parlow A. F Fed. Proc. 17 402 (1958)

W H L Hackeng R M Lequin W Schopman and H P M Persoon *Separation of radioactively labelled and un labelled proteinhormones by gelfiltration*

Laboratory for Endocrinological Chemistry Gemeenteziekenhuis Bergweg Rotterdam The Netherlands

The specific activity of radioactively labelled proteins is calculated from the quotient of the number of μC labelled protein and the quantity of protein used for labelling Thus an equal distribution from the label over the protein molecules is assumed

Gelfiltration of the labelled protein gives a number of fractions with a low percentage damage. When the same quantity of radioactivity from these tubes is incubated with a specific antibody often different B F ratios are obtained.

To investigate this phenomenon the elution pattern of ^{131}I glucagon and of unlabelled glucagon were studied.

With Sephadexgel (G-75) the topfraction of ^{131}I-glucagon is eluted before the unlabelled glucagon in albumin containing buffer

The same phenomenon was observed with ^{131}I-porkinsulin and unlabelled insulin at pH 3 whereas a reversal of the relative elution pattern was obtained at pH 7 6

The difference in B-F ratios obtained is thus explained by an increase in specific activity of these labelled hormones in some of the tubes.

P R Bouman and A Coort *Studies on the binding of insulin by rat diaphragm in vitro* To be published elsewhere

H Feenstra and J v d Vies *The influence of allylestrenol progesterone and oestradiol on the placenta of the rat*

Endocrinology Research Dept N V Organon Oss The Netherlands

Progesterone and allylestrenol increase the weight of the placenta in pregnant ovariectomized rats This effect is not found in normal pregnant rats or in ovariectomized rats to which a sufficiently high dose of oestradiol is administered together with the allylestrenol

From these experiments it appears that progesterone increases the growth of the placenta while oestrogen inhibits it. This suggests that the ovary by means of its progesterone and oestrogen production has a very profound regulating effect on the process of placental growth in normal rats.

C A de Groot *The effects of oestrogen or trifluoperazine pretreatments on the Parlow assay*

Department of Pharmacology University of Leiden The Netherlands

In PARLOW's (1958) ovarian ascorbic acid depleting assay for estimating luteinizing hormone (LH) in immature pseudopregnant recipients Wistar rats vary in control values as well as in reactions to doses of less than 1 μg purified LH Since prolactin stimulates ovarian activity in this respect the following pretreatments intended to enhance prolactin levels, were tried out

a) a single injection of 50 μg oestradiol benzoate (OB) 2 days before the assay
b) 3 consecutive daily doses of 1 mg trifluoperazine HCl (partly neutralized to pH 5) before the assay followed by ½ mg early on the day itself (NOVELLA *et al* 1965)
c) a course of low doses of OB (5 μg) which is started 3 days before the assay

This course can be extended, with the recipient rats remaining pseudopregnant and able to react to LH for some weeks (KIRCHER *et al* 1965)

those previously obtained in boys (HUIS IN 'T VELD 1960). The possible causes of changes which occur in the 17 kS excretion during the first two decades of life, are discussed.

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HUIS IN 'T VELD L. G. Maandschrift voor Kindergeneeskunde 28; 398 (1960).

Joh Koudstaal A. E. Boll and M. J. Hardonk *An investigation of the adrenal cortex of various mammals by histochemical and enzyme histochemical methods*

Department of Pathology Oostersingel 63 Groningen, The Netherlands

The adrenal cortex of the human and of a number of 10 mammals had been comparatively studied by histochemical and enzyme histochemical methods. The applied enzyme histochemical methods can be devided into 3 groups

I. *Enzymes directly related to steroidogenesis*
 3β-ol Hydroxysteroid dehydrogenase
 Secondary alcohol dehydrogenase

II. *Enzymes possibly related to steroidogenesis*
 NADH and NADPH tetrazolium reductases
 Glucose-6-phosphate and isocitric acid dehydrogenase
 Non-specific esterases (substrate α-naphthyl acetate, naphthol AS-D acetate and 4Cl-5 Br-indoxyl acetate)

III. *Enzymes not related to steroidogenesis*
 Alkaline and Acid phosphatase
 ATP-ase and 5-Nucleotidase
 Aminopeptidase
 Lactic acid and β-Hydroxybutyric acid dehydrogenase
 Succinic dehydrogenase and α Glycerophosphate oxidase

Generally the activity of the enzyme 3β-ol hydroxysteroid dehydrogenase is weak in the zona glomerulosa. In this zone there is no activity of the enzyme secondary alcohol dehydrogenase.

H Houtzager H van Leusden and J L Mastboom
*Conversion of androgens into estrogens by hydatidiform moles**

University Department of Obstetrics and Gynecology St Radboud Ziekenhuis Nymegen The Netherlands

Previous work from our laboratory showed that gram amounts of molar tissue incubated *in vitro* for two hours synthesized estrone and estradiol in high yields from labelled dehydroepiandrosterone (1–3) Hydatidiform moles lack the enzymes to convert C_{21} to C_{19} steroids.

Experiments are now reported on estrone and estradiol production using $^{4}\Delta$-androstenedione-^{14}C and testosterone-^{3}H as substrates. The products were isolated and purified by chromatography on thin layer plates, by recrystallization and by the formation of derivatives.

By experiments with $^{4}\Delta$-androstenedione-^{14}C testosterone-^{3}H added simultaneously and determination of the $^{14}C/^{3}H$ ratios of the purified products the reaction sequence of this metabolic pathway is elucidated.

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L G Huis in t Veld *Urinary excretion of individual neutral 17 ketosteroids in normal girls*

National Institute of Public Health, Utrecht The Netherlands

In 65 girls, 69 chromatographic analyses were made of the urinary 17 KS mixture The quantitative data obtained were statistically analysed in an attempt to establish the 'normal excretion' in females aged 0–20 years. The data obtained were compared with

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* Aided by a grant of the National Institutes of Health, Bethesda Md., U.S.A.

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  Lactic acid and β Hydroxybutyric acid dehydrogenase
  Succinic dehydrogenase and α Glycerophosphate oxidase

Generally the activity of the enzyme 3β-ol hydroxysteroid dehydrogenase is weak in the zona glomerulosa. In this zone there is no activity of the enzyme secondary alcohol dehydrogenase

The activity of this enzyme can be found in the zona reticularis and sometimes in the zona fasciculata. There is a great variability in intensity and localisation of the enzymes of group I in the various mammals There is a strong activity of the enzymes of group II in the zona glomerulosa, exceptionally the zona glomerulosa of the rat The division of the non-specific esterases is variable Enzymes of group III can be used to get a better idea of the functional morphology of the adrenal cortex There are sex differences in the mouse in the distribution of the enzymes alkaline phosphatase and succinic acid dehydrogenase

The results of this investigation indicate that several steps in steroidogenesis can take place in various parts of the adrenal cortex These parts are not the same in the adrenal cortex of the various mamals.

H G Kwa F Verhofstad and E M van der Bent

Radioimmunoassay of mouse prolactin based upon a protein isolated from prolactin-producing pituitary tumours

Department of Biology Antoni van Leeuwenhoek huis The Netherlands Cancer Institute, Amsterdam The Netherlands

Recently the isolation of rat prolactin (RP) from the granular fraction of transplants of pituitary tumours has been described (KWA VAN DER BENT and PROP 1967) and a radioimmunoassay based on ^{125}I labelled RP and an antiserum to RP has been reported briefly (KWA and VERHOFSTAD 1967)

Employing the same isolation technique a few mg of a protein were obtained from the granular fraction of first passage transplants of oestrogen induced prolactin producing pituitary tumours in mice The electrophoretic behaviour of this Mouse Pituitary Tumour Protein (MPTP) on polyacrylamide gels was comparable to that of bovine ovine and rat prolactin as is the case with RP it moved faster towards the anode at pH 8 0 than its homologous albumin

3mg MPTP were used to induce an antiserum in a rabbit The behaviour of MPTP in a radioimmuno system was then studied by labelling MPTP with ^{131}I by the Chloramine-T method (GREENWOOD HUNTER and GLOVER, 1963) and titrating four antisera

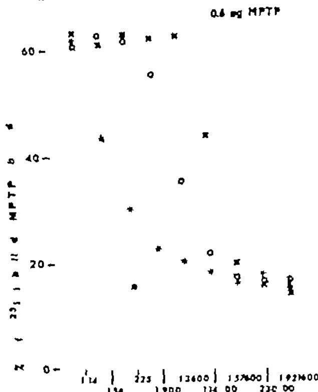


Fig. 1. Titration of antiserum to mouse proteins ( ), antiserum to swine prolactin (* *), antiserum to rat prolactin (•) and antiserum to MPTP (× ×) with ¹²⁵I labelled MPTP 0.05 ml of the four antisera in the indicated nine dilutions were allowed to equilibrate with 0.6 nanogram (ng) labelled MPTP in 0.10 ml diluent per vial. The 36 reaction mixtures were kept for 7 days at 4 °C. Aliquots (0.02 ml) were applied on cellulose acetate strips (OXOID 2.5cm × 20 cm) and subjected to electrophoretic separation (0.025 M Veronal buffer pH 8.6; 15 cm bridge, constant voltage of 300 V measured over electrodes, 2 h run). The strips were cut into four pieces 2 pieces of 1 cm on each side of the application side and 2 pieces of 9 cm, which were rolled into small scrolls. Radioactivity was measured in polyethylene counting vials in a PACKARD Autogamma Spectrometer (settings gain 90 %, window 20 to 70 keV). When counts on any of the two 1 cm pieces exceeded 20 % of the total count of the applied sample, excessive smearing was concluded to have taken place and another electrophoretic run for that sample was made.

Percentage of labelled antigen bound by antibody was calculated by dividing the radioactivity which had moved to the cathode by the total radioactivity applied.

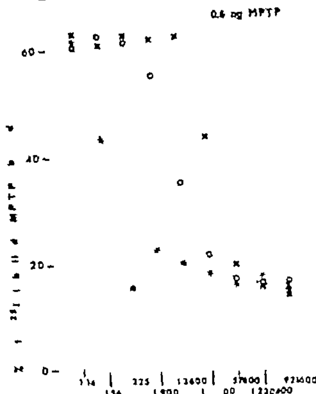


Fig. 1 Titration of antiserum to mouse proteins ( ) antiserum to ovine prolactin (* *) antiserum to rat prolactin ( ) and antiserum to MPTP (× ×) with 125I-labelled MPTP 0.05 ml of the four antisera in the indicated nine dilutions were allowed to equilibrate with 0.6 nanogram (ng) labelled MPTP in 0.10 ml diluent per vial. The 36 reaction mixtures were kept for 7 days at 4 °C Aliquots (0.02 ml) were applied on cellulose acetate strips (OXOID 2.5cm × 20 cm) and subjected to electrophoretic separation (0.025 M Veronal buffer pH 8.6; 15 cm bridge, constant voltage of 300 V measured over electrodes, 2 h run). The strips were cut into four pieces 2 pieces of 1 cm on each side of the application side and 2 pieces of 9 cm, which were rolled into small scrolls. Radioactivity was measured in polyethylene counting vials in PACKARD Autogamma Spectrometer (settings gain 80 %; window 20 to 70 keV). When counts on any of the two 1 cm pieces exceeded 20 % of the total count of the applied sample, excessive smearing was concluded to have taken place and another electrophoretic run for that sample was made.

Percentage of labelled antigen bound by antibody was calculated by dividing the radioactivity which had moved to the cathode by the total radioactivity applied.

raised to different antigens with 0 6 nanogram (ng) amounts of labelled MPTP in the reaction mixtures The reaction mixtures were allowed to equilibrate at 4 °C during 7 days the percentage of labelled MPTP bound by antibody in each reaction mixture was determined by electrophoretic separation of bound and free MPTP on cellulose acetate strips (HUNTER and GREENWOOD 1963) The titration curves are represented in Fig 1 The two antisera respectively to ovine and rat prolactin preparations showed a maximum binding of the labelled MPTP of around 63 % whereas the antiserum to mouse serum proteins failed to show any 'binding over and above 18 % Since binding by the three other antisera taper off to around 18 % binding at the higher dilutions, the observed minimal 'binding may be ascribed to incubation damage to the labelled hormone.

RÜMKE and LADIGES (1965) observed cross reactivity between an antiserum to ovine prolactin and mouse prolactin with the immunofluorescent technique although the gel double diffusion technique failed to disclose any cross reactivity between ovine and murine prolactin They presumed that mouse prolactin and sheep prolactin have only few antigenic determinants in common not enough to give rise to insoluble aggregation complexes, but sufficient for fixation of antibodies to the hormone in tissue sections. Employing an antiserum to rat prolactin an incomplete antigenic identity between prolactin from rat and mouse sources was demonstrated by the gel double diffusion technique (KWA VAN DER BENT and PROP 1967)

The results represented in Fig 1 confirm these observations the increase in percentage of labelled MPTP bound by antisera to ovine prolactin and antiserum to rat prolactin respectively from 18 % to 63 % indicates that at least 45 % of the labelled MPTP must have been bound by antibodies to known prolactin preparations and thus represent antigenically intact mouse prolactin or at least a murine protein that is antigenically closely related to prolactin The remainder of the radioactivity which fails to be bound by the antisera, must either have been an antigenically inert contaminating protein (unlikely) or antigenically damaged mouse prolactin since maximal binding by antiserum to MPTP itself is also around 63 %

Inhibition of the binding reaction between labelled MPTP and

antiserum to MPTP (0 6 ng labelled MPTP and 0 05 ml anti serum to MPTP in 1 2600 dilution in each reaction vial) was studied by adding 0 05 ml of increasing dilutions of mouse[1] anterior lobe tissue homogenates and of various mouse plasma samples, respectively Mouse anterior lobe tissue homogenates invariably showed good inhibition of the binding reaction at the appropriate

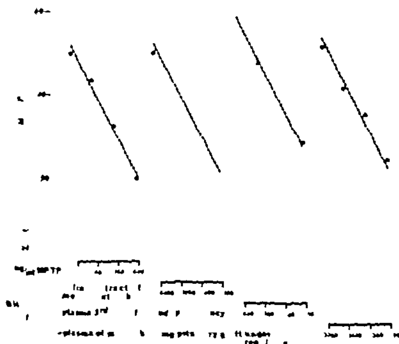


Fig. 2 Inhibition of the ^{125}I-labelled MPTP – antiserum to MPTP system 0.05 aliquots of antiserum in 1 2600 dilution were incubated with 0.6 ng labelled MPTP in 0.5 ml diluent in small reaction vials, to which 0.05 ml aliquots of the indicated dilutions of 1 MPTP standard, 2 saline extract of untreated male mouse pituitary glands, 3 plasma of a female mouse bearing an isologous pituitary gland under the renal capsule were added respectively Other data as in Fig 1

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[1] $(C_{57}BL \times CBA)F_1$ hybrid mice were used as donor mice for anterior lobe tissue and for plasma samples (0 1 ml of blood obtained by puncture of the ocular venous plexus).

dilutions Plasma from untreated male mice and from female mice collected in Dioestrous the first two stages of Pro-oestrus, during the second stage of Oestrus and all the stages of Metoestrus (defined according to THUNG BOOT and MÜHLBOCK, 1956) failed to inhibit the binding of labelled MPTP by antiserum under these conditions. However plasma samples collected during Pro-oestrus stages 3 and 4 or in Oestrus stage 1 showed inhibition in dilutions 1 9 and 1 30 Plasma samples from pseudo-pregnant mice showed marked inhibition occasionally up to dilutions of 1 640 whereas plasma from female mice bearing isologous pituitary gland tissue transplanted under the renal capsule for more than a year exhibited inhibition of the binding reaction up to dilutions of 1 6000 These results strongly suggest that the protein responsible for the inhibition by pituitary tissue extracts and by mouse plasma is prolactin

The four inhibition curves represented in Fig 2 show the inhibition obtained by 1 unlabelled MPTP 2 a saline (10 mg/ml) normal mouse anterior lobe tissue 3 plasma from a mouse on the third day of pseudo-pregnancy and 4 plasma from a mouse 18 months after transplantation of an isologous pituitary gland under the renal capsule The lines are parallel indicating antigenic identity as far as radioimmunoassay is concerned between MPTP and mouse prolactin It may therefore be concluded that radioimmunoassays employing labelled MPTP are valid when comparing prolactin in pituitary tissue extracts and in plasma and that the values obtained may be expressed in ng MPTP-standard

We are grateful to Dr L M BOOT for allowing us to collect blood samples from mice with pituitary isografts and to Professor O MÜHLBOCK for his continuous interest and active support.

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R M Lequin W Schopman W H L Hackeng and H P M Persoon *Some aspects of the radioimmunochemical determination of Parathyroid Hormone*

Laboratory for Endocrinological Chemistry Bergwegziekenhuis 62 Bergselaan, Rotterdam, The Netherlands

In order to measure Parathyroid Hormone in human plasma an extremely sensitive radioimmunoassay is necessary (BERSON and YALOW 1966) Purification of bovine ^{131}I PTH (iodination procedure according to GREENWOOD *et al.*) was troublesome. The relatively simple purification by means of Sephadex filtration – used for preparing insulin or human growth hormone tracer – gave dissatisfying results, i.e. the PTH-tracer gave incomplete binding to antibody excess although damage during incubation was low

Purification of the tracer was achieved by means of antibody binding (SCHOPMAN *et al* 1967) and resulted in a ^{131}I PTH tracer which could be used in a sensitive assaysystem (antiserum conc. 1 3000)

Sensitivity of the system could be further increased by addition of 1 M urea to the incubation buffer The smallest amount of added bovine PTH detectable is 10–20 pg

It was found that during immunisation with either highly purified or crude bovine PTH in guinea pigs, the mean association-K values of the antibodies increased (10^{8}–10^{9} L/M) they were however lower than those found for anti insulin, glucagon and human growth hormone antibodies (10^{10}–10^{11} L/M).

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J H van Maanen and P G Smelik *Depletion of monoamines in the hypothalamus and prolactin secretion*

Department of Pharmacology Medical Faculty University of Utrecht The Netherlands

The hypothalamus is supposed to elaborate a factor which inhibits prolactin secretion. Administration of reserpine or α

methyl-dopa induces pseudopregnancy indicating that prolactin has been released It is conceivable that this effect is due to depletion of hypothalamic catecholamines

Accordingly in female rats reserpine was implanted stereotaxically in the hypothalamus In this way a local depletion of monoamines could be achieved which was checked by means of a histochemical fluorescence method Reserpine implantation resulted in pseudopregnancy as judged from the disappearance of the estrous cycle and the occurrence of deciduoma formation

This effect of reserpine implantation could be prevented by administration of a MAO inhibitor indicating that the monoamine depletion was the causal factor

Z S Madjerek *Comparison of the traumatic deciduoma with the normal decidual reaction in mice*

Anat -embryol Lab University of Amsterdam The Netherlands

The endometrium of the laboratory rodents, under the influence of progesterone – either in a natural (pregnancy and pseudopregnancy) or in an artificial (conditioned by progesterone administration) progestational phase – reacts to a traumatization with the formation of deciduomata (LOEB 1908) Traumatic deciduoma formation in spayed mice is therefore an adequate method for testing progestational activities (MADJEREK 1960) It was shown earlier (KREHBIEL, 1937 SELYE and McKEOWN 1935) that the histological picture of a traumatic deciduoma in rats and in guinea pigs does not differ from that found in the uterus of these species during implantation We compared the histological preparations of traumatic deciduomata in the uteri of spayed progesterone-treated mice (MADJEREK 1960) with those of intact mice in the beginning of a normal pregnancy (5–7 days post coitum) This comparison showed that in mice too these reactions are histologically identical so that they are practically indistinguishable

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W Schopman W H L Hackeng R M Lequin and H P M Persoon *The glucagon content in tissues of the human intestinal tract*

Laboratory for Endocrinological Chemistry Gemeenteziekenhuis Bergweg Rotterdam The Netherlands

With radio-immunochemical methods different authors (SCHOPMAN 1965 SAMOLS *et al* 1966 UNGER *et al* 1966) proved the presence of glucagon in tissues of the gastro-intestinal tract. Mobilisation of this gut-glucagon could explain the higher insulin response of the pancreas after oral glucose administration compared to the intravenous route

The estimation of glucagon in pieces of tissue was extended with the measurement of the recovery of added radio-iodinated glucagon. The extracts were purified by dialyses and in some cases by dialyses and gelfiltration

Glucagon from the intestinal tract showed a behaviour identical to unlabelled standard glucagon The elution pattern of unlabelled glucagon and radio-iodinated glucagon found by gel filtration (Sephadex G-75) is not identical however In tissues of the gastro-intestinal tract obtained at autopsy within seven hours after death parallellism between extract and standard responses in the radio-immunoassay could not be rejected

When the mean glucagon content per gram of tissue – estimated for four autopsies – was multiplied by the organ weight – measured in one case only – the following glucagon contents in microgram per organ were calculated

oesophagus 1 anthrum 2 fundus 4 pylorus 0 3 duodenum 2 jejunum 13 ileum 57 colon 55 (for organ weights of 35 40 05 [illegible] 40 200 440 300 gram respectively) Moreover one third of the extrapancreatic tissue glucagon was found in liver

REFERENCES

[illegible] W., Results presented at the Boerhaave Course on *Diabetes* [illegible] (1965)

[illegible] J T[illegible] [illegible] and V MARKS, Lancet 2 727 (1966)

[illegible] and A. M. EISENTRAUT Metabolism 15

H B Schreuder and W Schopman *Influence of a rapid intravenous injection of glucose or tolbutamide on plasma insulin concentration in normal and diabetic subjects*

Department of Medicine & Lab of Clin. Chemistry O L V G Amsterdam Lab. of Endocrinological Chemistry Municipal Hospitals Rotterdam

The effect of a rapid intravenous injection of glucose (0 33 g/kg) on the plasma insulin level was studied in 9 young normal subjects (aged 24–36), in 9 elderly subjects (aged 50–73) and 16 patients with maturity-onset" diabetes (aged 47–70).

From all subjects a glucose disappearance coefficient (K_g) was calculated. Determination of plasma-insulin concentration (immunoassay) was performed in venous plasma, at 0-2-5-15-30-60-120 minutes after the start of the glucose injection.

In the elderly non-diabetic subjects the K_g values were significantly lower compared with the young normal subjects ($P < 0{,}05$) in all normal subjects the highest insulin concentration was found within 2–5 min after the glucose-injection.

The initial plasma-insulin rise in elderly non-diabetics did not differ significantly from that of the young normal group

The initial plasma insulin response to glucose of diabetic subjects was markedly impaired and delayed, when compared with the normal subjects.

In the normal subjects as well as in the diabetic patients no correlation has been found between K_g and plasma-insulin rise.

In the 9 elderly subjects and 15 diabetic patients the influence of a rapid intravenous injection of tolbutamide on the plasma-insulin concentration has been studied.

The results were compared with the effect of an intravenous injection of glucose.

P G Smelik and F v d Bilt *The effect of reserpine implantation in subcortical structures on ACTH secretion and on locomotor activity*

Department of Pharmacology Medical Faculty University of Utrecht The Netherlands

Implantation of reserpine in the hypothalamus of the rat results

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in depletion of the monoamines accumulated in the median eminence region as evidenced by the absence of fluorescent material (representing monoamines) after treatment with formaldehyde. In reserpine-implanted animals ACTH hypersecretion following administration of reserpine or chloropromazine is as usual indicating that these drugs do not induce ACTH release via an action on hypothalamic monoamines.

Other stressful stimuli also provoke the usual ACTH release in rats bearing hypothalamic reserpine implants.

Since in addition these animals are not sedated one may conclude that depletion of hypothalamic monoamines does not result in pituitary adrenal or behavioral deficiencies. It is possible however that the sedative and ACTH releasing effects of systemically administered reserpine are located somewhere else in the CNS Accordingly systematic reserpine implants were made at several sites in limbic structures and their effect on locomotor activity in an open field situation and on pituitary adrenal activity was tested. Implants were made bilaterally in the amygdala complex several areas of the hippocampus the caudate nucleus, the lateral septum the area preoptica, the reticular formation and the mammillary bodies. The results show that none of these implants when compared with sham implants, induced a significant effect on locomotor activity or ACTH secretion

It is concluded that there is no evidence that the action of reserpine is strictly localized in a specific subcortical structure This suggests the possibility that a much more extensive portion of the CNS is involved

A B Steffens *Blood glucose levels and food intake in normal and hypothalamic hyperphagic rats*

Zoological Laboratory University of Groningen The Netherlands

Like other mammals an adult rat kept under ad libitum conditions maintains a fairly constant body weight during prolonged periods, provided that its hypothalamus is intact Destruction of the ventromedial hypothalamic nuclei causes hyperphagia resulting in obesity lateral hypothalamic lesion on the other hand induces aphagia.

In his glucostatic theory J Mayer has postulated that the individual regulates its food intake in accordance with the availability of blood glucose (defined in terms of the difference (ΔV) between arterial and venous glucose concentrations) low availability activating and high availability inhibiting feeding behaviour The regulating signals arising from glucose-availability would then be processed by the hypothalamus. Although there are some indications in the literature that the occurrence of feeding behaviour shows some overall correlating with ΔV there is little proof that low availability is a direct cause of the initiation and high availability of the termination of intake

To check this one would need more detailed data on the time course of ΔV than are available so far A major difficulty here is that arterial blood glucose levels fluctuate rapidly so that little reliance can be put on ΔV determinations when the time interval between the arterial and venous sample on which they are based is unspecified and possibly quite arbitrary

As a first step towards a re-examination of these questions I have therefore begun to relate arterial blood glucose levels to food intake in rats under different experimental conditions. In addition, I have determined the concentration of free fatty acids (FFA), the other major substrate for energy metabolism. For these purposes, I have developed a technique for blood sampling in the rat by means of a chronic intravenous cannula. This permits taking off an unlimited number of samples (blood withdrawn being replaced by equivalent transfusion) over a period of many weeks. Sampling never causes any observable disturbance in the ongoing behaviour (feeding, sleeping, etc.) of the subject. Further by means of a double cannula simultaneous sampling and infusion were made possible Glucose concentration is determined by means of a Technicon Auto Analyzer FFA levels by the titrimetric method of Meinertz and Dole So far blood samples have been taken either at long (2 h) intervals or at short intervals (4 min during meals and 10 min in between meals) The results obtained up to the present are as follows

1 After 4 h fasting, glucose levels are low and FFA levels high, as compared to the *ad libitum* condition. If food is returned to the fasted animal, the *ad libitum* situation is reinstated in the blood within fifteen minutes.

2. Glucose infusions (1 2 ml/h of a 50 % solution) administered to the fasted animal induce the same lowering of FFA levels as does presentation of food. In contrast infusion of a 20 % fat emulsion at the same rate does not influence blood FFA. It is concluded that the FFA changes are caused (via an undetermined chain of processes) by changes in blood glucose
3 FFA levels fluctuate widely under *ad libitum* conditions. These fluctuations bear no relation to feeding behaviour
4 In the *ad libitum* animal glucose concentration in between meals is fairly constant it rises rapidly within a few minutes after the start of a meal and returns to pre-meal values soon after its termination
5 If the food is removed at the moment that the rat starts a meal a slow but very prolonged fall in glucose level ensues.

These results seem compatible with the view that changes in blood glucose level set up signals that are a (contributory) cause both of the initiation and termination of feeding behaviour This, of course is not to say that they constitute proof of such a view Further work is in progress including a study of certain endocrine aspects, to establish whether it will remain tenable

In the ventromedial hypothalamic hyperphagic rat blood glucose levels in the dynamic phase under *ad libitum* conditions are not different from those in the intact subject under similar conditions in between meals. These lesioned animals eat huge meals at regular intervals, but in many cases these meals have hardly any effect on blood glucose It seems plausible to ascribe this finding to the fact that in the dynamic hyperphagic rat the gut is full at all times so that digestion and insulin production are activated continuously The hyperphagia of the lesioned subject could then be due to its inability caused by the lesion to take satiety signals arising from blood glucose into account. FFA levels in the lesioned rats appear similar to those of the intact animal in every respect.

I E Uyldort and J M Villaudy *The development of mammary gland from pregnant rats in organ culture*

Laboratory of Pharmacology University of Amsterdam The Netherlands

Sterile fragments (1 5–2.0 mm) of mammary gland taken from

rats on the 9th–17th day of pregnancy were cultured at 37 °C at pH 7.5–8.3 on rayon acetate strips on the surface of a medium consisting of 50 % rat serum and 50 % Trowell medium (T 8) (from which the amino-acids, phenol red, chloramphenicol and *p-amino benzoic* acid were omitted), containing 50 µg/ml insulin, final concentration. The serum was always taken from rats 3 days further advanced in pregnancy. On changing the medium (every 3 days) serum was used from rats each time advanced another 3 days in pregnancy.

Culturing for 6 days with insulin only resulted in proliferation of the acini, which appear compact and show many mitoses. *Addition of progesterone (P) 0.1–0.5 µg/ml as well as oestradiol* (Oe) 0.01–0.05 µg/ml causes vacuolisation of the acinal cells. Further addition of hydrocortisone (H) 4–20 µg/ml causes extensive secretion, the histological picture being strikingly similar to that shown *in vivo* by a mammary gland late in pregnancy.

Culturing for 6 days with insulin only followed by 3 days with insulin plus P plus Oe, plus H, plus ovine prolactin (Prol) (10 µg/ml) plus bovine growth hormone (GH) (10 µg/ml) showed mainly proliferation. 3 Days insulin only followed by 6 days under further addition of P plus Oe plus H, plus Prol, plus GH resulted in abundant secretion.

J. de Visser: *Some aspects of the relation between chemical structure of steroids and their prostatotropic effect in rats*

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The androstane derivative $\Delta^{8,14}$-5αH-androstadiene-3,17-diol, 3,17-di-acetate (NB 06) displayed a preferential increase of ventral prostate weight in young normal and castrated rats after daily subcutaneous administration for two weeks. According to Saunders (1963) the quotient

$$\frac{\text{ventral prostate weight}}{\text{seminal vesicle weight}}$$

($Q_{VP/SV}$) is useful to indicate a difference in androgen sensitivity of both organs.

All hormone preparations were kindly provided by N.V. Organon Factories, Oss, The Netherlands.

Increasing doses of testosteronepropionate (tep) gave low Q-values as measured in the Hershberger test. After treatment with increasing doses of NB 06 higher Q values could be calculated which were positively correlated with the doses administered The difference between the androgenic activity of tep and NB 06 in castrated rats is that the latter has no or only a very weak growth effect on the seminal vesicles.

Oestradiol could not abolish the prostatotropic effect of NB 06 and on the other hand NB 06 could not inhibit growth of the seminal vesicles induced by oestradiol

NB 06 like tep could partly maintain testis weight in hypophysectomized hemi-castrated rats.

Furthermore NB 06 did not inhibit the function or development of the gonads in young male and female rats.

A weak preferential prostatotropic effect could be demonstrated in castrated mice after NB 06 treatment.

As measured in the Hershberger test some androstane compounds, chemically related to NB 06 displayed varying effects showing remarkable connection between chemical structure and pharmacological activity

J D Wiener *The presence of iodotyrosine-like compounds in normal serum*

Department of Internal Medicine Free University Hospital Amsterdam The Netherlands

After the administration of *radioactive* iodine to normal animals or human subjects labelled thyroxine but at most traces of the precursors mono- and diiodotyrosine (MIT and DIT) can be detected in the serum However several investigators claim to have found considerable amounts of MIT and DIT when assaying the iodinated com ounds in serum with *chemical* methods We have previously reported the presence of labelled iodotyrosines (notably DIT) in the serum of rats brought to isotopic equilibrium with ^{131}I iodotyrosines were present in those groups which received a sufficient amount of iodine in the diet but hardly in iodine deficient groups (WIENER, 1965)

To explain these findings, we proposed a schematic model of the thyroidal iodine metabolism based on (a) the hypothesis that the iodotyrosines are present in the circulation in a "masked form (i.e. protected against deiodination both inside and outside the thyroid) and (b) the – known – functional heterogeneity of the thyroid tissue. According to this model the functionally active part of the gland would elaborate the hormone while the relatively inactive part – very much more slowly – would synthesize and release masked iodotyrosines. The latter would be unmasked" during chemical isolation and therefore be determined as iodotyrosines. The proportion of inactive follicles increases with the iodine intake and is low in long-standing severe iodine deficiency this would explain the difference in circulating DIT found in rats on different diets.

We now have the means to determine, be it indirectly the circulating iodotyrosine-like compounds chemically (BACKER *et al.*, 1967). An experiment was set up with healthy volunteers in Amsterdam where the iodine intake though not very low is certainly suboptimal Serum was analysed before during and after three consecutive periods of ten to fifteen weeks when the subjects took 200 600 and 1800 μg KI per day respectively

The results of this study are presented

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J Zweens and P R Bouman *Neoformation of insulin producing islets following ligation of the pancreatic ducts in normal and alloxan-diabetic rats*

Department of Pharmacology University of Groningen The Netherlands

Formation of new islets of Langerhans in adult mammals of various species has been described on microscopic examination in a variety of experimental conditions, including prolonged hyper glycemia, and the administration of alloxan. However the cell type from which these new islets develop has remained a matter

of discussion whereas no information is available on the functional activity of newly formed islet tissue

The present investigation deals with the effect of ligation of the excretory duct in the distal or splenic part of the pancreas on its endocrine apparatus. Microscopic examination of pancreas distal to the ligature revealed that apart from degeneration of the exocrine tissue ligation of the pancreas gives rise to the development of a considerable number of islet-like structures. This is in accordance with almost forgotten data from BENSLEY (1915) on the rabbit and GRAUER (1926) *on the guinea pig The presence of beta-cells* in these structures was demonstrated by selective colouring with aldehyd fuchsin (GOMORI-SCOTT 1952) *Examination of serial* sections revealed that the Gomori positive tissue derives from the exocrine pancreatic ductules by budding and transformation of ductal elements into beta-cells. Accordingly it was concluded that neoformation of islet tissue occurs in response to ligation This phenomenon occurs from the third day after operation on.

In order to determine the insulin content of the pancreas extractions were performed with the technique described by DAVIDSON *et al* (1963) Separate extracts were made from the parts proximal and distal to the ligature one and four weeks after ligation and from corresponding parts of intact control pancreas. The insulin like activity (ILA) of these extracts was determined using a rat epididymal fat pad assay (STEELMAN *et al* 1960) whereas immunoreactive insulin (IRI) was determined by radioimmunoassay (YALOW and BERSON 1960) One week after ligation a significant rise in the amount of extractable ILA and IRI was observed in the distal part of the pancreas, while four weeks after ligation a twofold rise in the amount of extractable ILA and IRI was found in both the proximal and the distal part of the pancreas. These findings indicate that insulin is being produced by the newly formed islet tissue

Its possible functional significance was investigated by subjecting alloxanized rats to ligation or sham-operation whereafter the course of the diabetes was followed Glucosuria was determined at weekly intervals from 1 to 5 weeks postoperatively In both groups the animals with an initial glucosuria higher than 5 g/24 h did not recover from their diabetic state during the period of observation However in animals with an initial glucosuria of

less than 5 g/25 h, complete remission was significantly more frequent in the group of ligated animals (60 %) than in the group of sham-operated animals (33 %).

It is concluded that in the rat ligation of the pancreas results in neoformation of insulin-producing islets which derive from exocrine pancreatic ducts. The high incidence of remission of diabetes in ligated alloxanized rats suggests a considerable functional activity of the newly formed islets.

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